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Jeff Lloyd, Patent Attorney, Reg. No. 35,589

REQUEST FOR CERTIFICATE OF CORRECTION UNDER 37 CFR 1.322
Docket No. MPS 11.83D2D1
Patent No. 7,345,229

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Michael J. Adang, John D. Kemp, Ebrahim Firoozabady
Issued	:	March 18, 2008
Patent No.	:	7,345,229
For	:	Insect Resistant Cotton Plants

Mail Stop Certificate of Corrections Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR CERTIFICATE OF CORRECTION
UNDER 37 CFR 1.322 (OFFICE MISTAKE)

Sir:

A Certificate of Correction for the above-identified patent has been prepared and is attached hereto.

In the left-hand column below is the column and line number where errors occurred in the patent. In the right-hand column is the page and line number in the application where the correct information appears.

Patent Reads:	**Application Reads:**
Column 1, line 10:	Page 1, line 8:
"Oct. 21, 1988"	--October 20, 1988--
Column 3, line 30:	Page 5, line 4:
"Ruvidn"	--Ruvkin--

Column 4, line 19:	Page 6, line 15:
"market"	--marker--
Column 8, line 39:	Page 13, line 19:
"Aidyoshi"	--Akiyoshi--
Column 9, line 32:	Page 15, line 2:
"one"	--*onc* --
Column 9, line 34:	Page 15, line 4:
"one"	--*onc*--
Column 10, line 10:	Page 16, line :
"one genes), and nine (having one"	--*onc* genes), and nine (having *onc*--
Column 10, line 28:	Page 16, line 17:
"one"	--*onc*--
Column 13, line 28:	Page 21, line 15:
"one"	--*onc*--
Column 39, line 22:	Page 64, line 20:
"BgI11"	--*Bgl*II--
Column 41, line 31:	Page 68, line 5:
"Bgm"	--*Bgl*II--
Column 41, lines 47-48:	Page 68, line 16:
"SmaI-lineanzed, M13 mp19 RF DNA"	--*Sma*I-linearized, M13mp19 RF DNA--
Column 42, line 54:	Page 70, line 9:
"7.5 mg/1 &"	--7.5 mg/16- --

Column 42, line 65:	Page 70, line 18:
"*B. thunngiensis*"	--*B. thuringiensis*--
Column 44, line 57:	Page 73, line 21:
"*Nicoliana tabacum*"	--*Nicotiana tabacum*--
Column 47, line 38:	Page 78, line 14:
"(SD Indicates the Location of a Shine-Dalgarno Prokaryotic Ribosome Binding Site.)"	--SD indicates the location of a Shine-Dalgarno prokaryotic ribosome binding site.)--
Column 48, line 48:	Page 80, line 5:
"Hind III"	--*Hind*III--
Column 49, line 12:	Page 81, line 3:
"pIC351A's"	--pIC35/A's--
Column 51, line 3:	Page 84, line 3:
"transcnbed"	--transcribed--
Column 51, line 4:	Page 84, line 3:
"CAMV"	--CaMV--
Column 52, line 46:	Page 86, line 23:
"$2.5x10^6$ ml^-."	--$2.5x10^6$ ml^{-1}.--
Column 53, line 2:	Page 87, line 11:
"thiamine. HC1 "	--thiamine · HC1 --
Column 53, line 30:	Page 88, line 4:
"caili"	--calli--

Column 54, line 22,	Page 89, line 17:
"0.50 mg/l "	--50 mg/l --
Column 54, line 63:	Page 90, line 18:
"*Agrobactetium*"	--*Agrobacterium*--
Column 55, line 4:	Page 90, line 24:
"6t1016"	--6t0016--
Column 55, line 38:	Page 91, 19:
"0.1 mlavell"	--0.1 ml/well--
Column 56, line 21:	Page 92, line 25:
"Was"	--was--
Column 56, line 55	Page 93, lines 20-21:
"*Manduca seta*"	--*Manduca sexta*--
Column 57, line 62:	Page 95, line 17:
"(UC82 Ro plants"	--(UC82 R_0 plants--
Column 58, line 57:	Page 97, line 4:
"25 mg/A"	--25 mg/l--
Column 59, line 5:	Page 97, line 15:
"axiliary"	--axillary--
Column 59, line 12:	Page 97, line 20:
"carbenicllin"	--carbenicillin--
Column 65, line 3:	Request for Reconsideration under 37 CFR 1.111 dated January 5, 2006, page 12:
"GRMg"	--GRM_{gn}--

Column 68, line 2:	Request for Reconsideration under 37 CFR 1.111 dated January 5, 2006, page 15:
"9 $\mu m^2/s$"	--9 $\mu Em^2/s$--
Column 71, line 8:	Request for Reconsideration under 37 CFR 1.111 dated January 5, 2006, page 19:
"G0"	--G_0--
Column 72, line 6:	Request for Reconsideration under 37 CFR 1.111 dated January 5, 2006, page 21:
"Embryopenic"	--embryogenic--
Column 72, line 34:	Request for Reconsideration under 37 CFR 1.111 dated January 5, 2006, page 21:
"(G I or G_3)"	--(G_1 or G_3) --
Column 73, line 14:	Request for Reconsideration under 37 CFR 1.111 dated January 5, 2006, page 22:
"(9 $gE/m^2/s$)"	--(9 $\mu E/m^2/s$)--
Column 76, line 55:	Page 101, line 14:
"Polpomyla sp."	--Polpomyia sp.--
Column 79, line 62:	Page 106, line 32:
"Phyllonotycter"	--Phyllonorycter--
Column 80, line 23:	Page 107, line 14:
"Spionota"	--Spilonota--
Column 80, line 36:	Page 107, line 29:
"Thaumetopoea pilyocampa"	--Thaumetopoea pityocampa--
Column 83, line 16:	Page 114, line 6:
"GaMV, Tn5"	--CaMV, Tn5--

Column 84, Table 7, Experiment 1^5, Column CP^2, Row 102:	Page 117, Table 7, Experiment 1^5, Column CP^2, Row 102:
"+"	-- ±--
Column 84, Table 7, Experiment 1^5, Column CP^2, Row 110:	Page 117, Table 7, Experiment 1^5, Column CP^2, Row 110:
"+"	-- ±--
Column 87, Table 14 foot note, lines 1-2:	Request for Reconsideration under 37 CFR 1.111 dated January 5, 2006, page 26:
"myo-inosi-tol"	--myo-inositol--

A true and correct copy of pages 1, 5, 6, 13, 15, 16, 21, 64, 68, 70, 73, 78, 80, 81, 84-93, 95, 97, 101, 106, 107, 114 and 117 of the specification as filed and Request for Reconsideration under 37 CFR 1.111, dated January 5, 2006 which support Applicants' assertion of the errors on the part of the Patent Office accompanies this Certificate of Correction.

The Commissioner is authorized to charge any additional fees as required under 37 CFR 1.20(a) to Deposit Account No. 19-0065.

Approval of the Certificate of Correction is respectfully requested.

Respectfully submitted,

Jeff Lloyd
Patent Attorney
Registration No. 35,589
Phone No.: 352-375-8100
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Address: P.O. Box 142950
Gainesville, FL 32614-2950

JL/jb

Attachments: Copy of pages 1, 5, 6, 13, 15, 16, 21, 64, 68, 70, 73, 78, 80, 81, 84-93, 95, 97, 101, 106, 107, 114 and 117 of the specification
Copy of Request for Reconsideration under 37 CFR 1.111, dated January 5, 2006

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,345,229

APPLICATION NO.: 08/478,153

DATED : March 18, 2008

INVENTOR : Michael J. Adang, John D. Kemp, Ebrahim Firoozabady

It is certified that errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 1,
Line 10, "Oct. 21, 1988" should read -- October 20, 1988--.

Column 3,
Line 30, "Ruvidn" should read -- Ruvkin --.

Column 4,
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Column 9,
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Column 10,
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Line 28, "one" should read --*onc* --.

Column 13,
Line 28, "one" should read --*onc* --.

MAILING ADDRESS OF SENDER:
Saliwanchik, Lloyd & Saliwanchik
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Line 22, "BgI11" should read --*Bgl*II--.

Column 41,
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Line 54, "7.5 mg/1 &" should read -- 7.5 mg/16- --.
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Column 47,
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Column 56,
Line 21, "Was" should read -- was--.
Line 55, "*Manduca seta*" should read --*Manduca sexta*--.

Column 57,
Line 62, "(UC82 Ro plants" should read -- (UC82 R_0 plants --.

Column 58,
Line 57, "25 mg/A" should read -- 25 mg/l --.

Column 59,
Line 5, "axiliary" should read -- axillary --.
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Column 65,
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Column 68,
Line 2, "9 $\mu m^2/s$ " should read –9 $\mu Em^2/s$ --.

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Column 72,
Line 6, "Embryopenic" should read -- embryogenic --.
Line 34, "(G I or G_3)" should read --(G_1 or G_3) --.

Column 73,
Line 14, "(9 $gE/m^2/s$)" should read -- (9 $\mu E/m^2/s$) --.

Column 76,
Line 55, "Polpomyla sp." should read -- Polpomyia sp.--.

Column 79,
Line 62, "Phyllonotycter" should read -- Phyllonorycter --.

Column 80,
Line 23, "Spionota" should read -- Spilonota --.
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Column 83,
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Column 84,
Table 7, Experiment 1^5, Column CP^2, Row 102, "+" should read -- $\pm$ --.

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Table 7, Experiment 1^5, Column CP^2, Row 110, "+" should read -- ±--.

Column 87,
Table 14 foot note, lines 1-2, "myo-inosi-tol" should read -- myo-inositol --.

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DESCRIPTION

Cotton
INSECT RESISTANT PLANTS

Cross-Reference to Related Applications

This application is a continuation of 08/151,615, filed November 12, 1993; which is a division of Serial No. 07/713,624, filed June 10, 1991; which is a file-wrapper-continuation of Serial No. 07/260,574, filed October 20, 1988; which was a continuation-in-part of Serial No. 06/848,733, filed April 4, 1986; which was a continuation-in-part of the first filed application in this chain, Serial No. 06/535,354, filed September 26, 1983, through which the benefit of priority is hereby claimed pursuant to 35 U.S.C. §120.

Field of the Invention

The present invention is in the fields of genetic engineering, plant husbandry, and bacterial bio-affecting compositions, especially those derived from the genus *Bacillus*.

Background of the Invention

Insecticidal Protein. *Bacillus thuringiensis*, a species of bacteria closely related to *B. cereus*, forms a proteinaceous crystalline inclusion during sporulation. This crystal is parasporal, forming within the cell at the end opposite from the developing spore. The crystal protein, often referred to as the δ-endotoxin, has two forms: a nontoxic protoxin of approximate molecular weight (MW) of 130 kilodaltons (kD), and a toxin having an approximate MW of 67 kD. The crystal contains the protoxin protein which is activated in the gut of larvae of a number of insect species. Klowden, M.J. *et al.* (1983) *Appl. Environ. Microbiol.* 46:312-315, have shown solubilized protoxin from *B. thuringiensis* var. *israelensis* is toxic to *Aedes aegypti* adults. During activation, the protoxin is cleaved into two polypeptides, one or both

Chang, S. (1983) *Trends Biotechnol.* 1:100-101, reported that the DNA sequence of a complete HD-1 gene had been publicly presented (ref. 5 therein), and that the HD-1 toxin moiety resides in the amino-terminal 67 kD peptide.

Shuttle Vectors. Shuttle vectors, developed by Ruvkin, G.B., F.M. Ausubel (1981) *Nature* 298:85-88, provide a way to insert foreign genetic materials into position of choice in a large plasmid, virus, or genome. There are two main problems encountered when dealing with large plasmids or genomes. First, the large plasmids may have many sites for each restriction enzyme. Unique site-specific cleavage reactions are not reproducible, and multi-site cleavage reactions followed by ligation lead to great difficulties due to the scrambling of the many fragments whose order and orientation one does not want changed. Second, the transformation efficiency with large DNA plasmids is very low. Shuttle vectors allow one to overcome these difficulties by facilitating the insertion, often *in vitro*, of the foreign genetic material into a smaller plasmid, then transferring, usually by *in vivo* techniques, to the larger plasmid.

A shuttle vector consists of a DNA molecule, usually a plasmid, capable of being introduced into the ultimate recipient bacteria. It also includes a copy of the fragment of the recipient genome into which the foreign genetic material is to be inserted and a DNA segment coding for a selectable trait, which is also inserted into the recipient genome fragment. The selectable trait ("marker") is conveniently inserted by transposon mutagenesis or by restriction enzymes and ligases.

The shuttle vector can be introduced into the ultimate recipient cell, typically a bacterium of the family Rhizobiaceae (which contains the genus *Agrobacterium*), by a tri-parental mating (Ruvkin and Ausubel, *supra*), direct transfer of a self-mobilizable vector in a bi-parental mating, direct uptake of exogenous DNA by *Agrobacterium* cells ("transformation," using the conditions of Holsters, M. *et al.* [1978] *Mol. Gen. Genet.* 163:181-187), by spheroplast fusion of *Agrobacterium* with another bacterial cell, by uptake of liposome-encapsulated DNA, or by infection with a shuttle vector that is based on a virus that is capable of being packaged *in vitro*.

A tri-parental mating involves the mating of a strain containing a mobilizable plasmid, which carries genes for plasmid mobilization and conjugative transfer, with the strain containing the shuttle vector. If the shuttle vector is capable of being mobilized by the plasmid genes, the shuttle vector is transferred to the recipient cell containing the large genome, e.g., the Ti or Ri plasmids of *Agrobacterium* strains.

After the shuttle vector is introduced into the recipient cell, possible events include a double crossover with one recombinational event on either side of the marker. This event will result in transfer of a DNA segment containing the marker to the recipient genome replacing a homologous segment lacking the insert. To select for cells that have lost the original shuttle vector, the shuttle vector must be incapable of replicating in the ultimate host cell or be incompatible with an independently selectable plasmid pre-existing in the recipient cell. One common means of arranging this is to provide in the third parent another plasmid which is incompatible with the shuttle vector and which carries a different drug resistance marker. Therefore, when one selects for resistance to both drugs, the only surviving cells are those in which the marker on the shuttle vector has recombined with the recipient genome. If the shuttle vector carries an extra marker, one can then screen for and discard cells that contain plasmids resulting from a single crossover event between the shuttle vector and the recipient plasmid resulting in cointegrates in which the entire shuttle vector is integrated with the recipient plasmid. If the foreign genetic material is inserted into or adjacent to the marker that is selected for, it will also be integrated into the recipient plasmid as a result of the same double recombination. It might also be carried along when inserted into the homologous fragment at a spot not within or adjacent to the marker, but the greater the distance separating the foreign genetic material from the marker, the more likely will be a recombinational event occurring between the foreign genetic material and marker, preventing transfer of the foreign genetic material.

If the shuttle vector is used to introduce a phenotypically dominant trait (e.g., a novel expressible insecticide structural gene, but not an inactivated oncogenic T-DNA gene), one need not only rely on a double homologous recombination. The

Roots resulting from transformation from *A. rhizogenes* have proven relatively easy to regenerate directly into plantlets (Chilton, M.-D. *et al.* [1982] *Nature* 295:432-434).

Agrobacterium – Genes on the TIP Plasmids. A number of genes have been identified within the T-DNA of the TIP plasmids. About half a dozen octopine plasmid T-DNA transcripts have been mapped (Gelvin, S.B. *et al.* [1982] *Proc. Natl. Acad. Sci. USA* 79:76-80; Willmitzer, L. *et al.* [1982] *EMBO J.* 1:139-146) and some functions have been assigned (Leemans, J. *et al.* [1982] *EMBO J.* 1:147-152). Some of these transcripts, specifically those in the region encoding *tmr* and *tms*, can also be transcribed in prokaryotic cells (Schroder, G. *et al.* [1983] *EMBO J.* 2:403-409). The four genes of an octopine type plasmid that have been well defined by transposon mutagenesis include *tms*, *tmr*, and *tml* (Garfinkel, D.J. *et al.* [1981] *Cell* 27:143-153). Ti plasmids which carry mutations in these genes respectively incite tumorous calli of *Nicotiana tabacum* which generate shoots, proliferate roots, and are larger than normal. In other hosts, mutants of these genes can induce different phenotypes (see Bevan, M.W., M.-D. Chilton [1982] *Ann. Rev. Genet.* 16:357-384). The phenotypes of *tms* and *tmr* are correlated with differences in the phytohormone levels present in the tumor. The differences in cytokinin:auxin ratios are similar to those which in culture induce shoot or root formation in untransformed callus tissue (Akiyoshi, D.E. *et al.* [1983] *Proc. Natl. Acad. Sci. USA* 80:407-411). T-DNA containing a functional gene for either *tms* or *tmr* alone, but not functional *tml* alone, can promote significant tumor growth. Promotion of shoots and roots is respectively stimulated and inhibited by functional *tml* (Ream, L.W. *et al.* [1983] *Proc. Natl. Acad. Sci. USA* 80:1660-1664). Mutations of T-DNA genes do not seem to affect the insertion of T-DNA into the plant genome (Leemans *et al.* [1982] *supra*; Ream *et al.* [1983] *supra*). The *ocs* gene encodes octopine synthase, which has been sequenced by De Greve, H. *et al.* (1982) *J. Mol. Appl. Genet.* 1:499-511. It does not contain introns (intervening sequences commonly found in eukaryotic genes which are post-transcriptionally spliced out of the messenger precursor during maturation of the mRNA). It does have sequences

Garfinkel, D.J., E.W. Nester [1980] *J. Bacteriol.* 144:732-743; and Ooms, G. [1980] *J. Bacteriol.* 144:82-91, for octopine plasmids). Most important are the *onc* genes, which, when mutated, result in Ti plasmids incapable of oncogenicity. (These loci are also known as *vir*, for virulence.) Several *onc* genes have been accurately mapped and have been found to be located in regions conserved among various Ti plasmids (Klee, H.J. *et al.* [1983] *J. Bacteriol.* 153:878-883; Iyer, V.N. *et al.* [1982] *Mol. Gen. Genet.* 188:418-424). The *onc* genes function in *trans*, being capable of causing the transformation of plant cells with T-DNA of a different plasmid type and physically located on another plasmid (Hille, J. *et al.* [1982] *Plasmid* 7:107-118); Klee, H.J. *et al.* [1982] *J. Bacteriol.* 150:327-331; de Framond, A.J. *et al.* [1983] *Biotechnol.* 1:262-269). Nopaline Ti DNA has direct repeats of about 25 base pairs immediately adjacent to the left and right borders of the T-DNA which might be involved in either excision from the Ti plasmid or integration into the host genome (Yadav, N.S. *et al.* [1982] *Proc. Natl. Acad. Sci. USA* 79:6322-6326), and a homologous sequence has been observed adjacent to an octopine T-DNA border (Simpson, R.B. *et al.* [1982] *Cell* 29:1005-1014). Opine catabolism is specified by the *occ* and *noc* genes, respectively, of octopine- and nopaline-type plasmids. The Ti plasmid also encodes functions necessary for its own reproduction including an origin of replication. Ti plasmid transcripts have been detected in *A. tumefaciens* cells by Gelvin, S.B. *et al.* (1981) *Plasmid* 6:17-29, who found that T-DNA regions were weakly transcribed along with non-T-DNA sequences. Ti plasmid-determined characteristics have been reviewed by Merlo, *supra* (see especially Table II), and Ream and Gordon, *supra*.

Agrobacterium – TIP Plasmid DNA. Different octopine-type Ti plasmids are nearly 100% homologous to each other when examined by DNA hybridization (Currier, T.C., E.W. Nester [1976] *J. Bacteriol.* 126:157-165) or restriction enzyme analysis (Sciaky, D. *et al.* [1978] *Plasmid* 1:238-253). Nopaline-type Ti plasmids have as little as 67% homology to each other (Currier and Nester, *supra*). A survey revealed that different Ri plasmids are very homologous to each other (Costantino, P. *et al.* [1981] *Plasmid* 5:170-182). Drummond, N.H., M.-D. Chilton (1978) *J.*

Bacteriol. 136:1178-1183, showed that proportionally small sections of octopine- and nopaline-type Ti plasmids were homologous to each other. These homologies were mapped in detail by Engler, G. *et al.* (1981) *J. Mol. Biol.* 152:183-208. They found that three of the four homologous regions were subdivided into three (overlapping the T-DNA), four (containing some *onc* genes), and nine (having *onc* genes) homologous sequences. The uninterrupted homology contains at least one *tra* gene (for conjugal transfer of the Ti plasmid to other bacterial cells), and genes involved in replication and incompatibility. This uninterrupted region has homology with a *Sym* plasmid (involved in symbiotic nitrogen fixation) from a species of *Rhizobium*, a different genus in the family Rhizobiaceae (Prakash, R.K. *et al.* [1982] *Plasmid* 7:271-280). The order of the four regions is not conserved, though they are all oriented in the same direction. Part of the T-DNA sequences is very highly conserved between nopaline and octopine plasmids (Chilton, M.-D. *et al.* [1978] *Nature* 275:147-149; Depicker, A. *et al.* [1978] *Nature* 275:150-153). Ri plasmids have been shown to have extensive homology among themselves, and to both octopine (White, F.F., E.W. Nester [1980] *J. Bacteriol.* 144:710-720) and nopaline (Risuleo, G. *et al.* [1982] *Plasmid* 7:45-51) Ti plasmids, primarily in regions encoding *onc* genes. Ri T-DNA contains extensive though weak homologies to T-DNA from both types of Ti plasmid (Willmitzer, L. *et al.* [1982] *Mol. Gen. Genet.* 186:16-22). Plant DNA from uninfected *Nicotiana glauca* contains sequences, referred to as cT-DNA (cellular T-DNA), that show homology to a portion of the Ri T-DNA (White, F.F. *et al.* [1983] *Nature* 301:348-350; Spano, L. *et al.* [1982] *Plant Mol. Biol.* 1:291-300). Huffman, G.A. *et al.* (1983) *J. Bacteriol.*, have mapped the region of cross-hybridization and have shown that Ri plasmid, pRiA4b, is more closely related to a pTiA6 (octopine-type) than pTiT37 (nopaline-type) and that this Ri plasmid appears to carry sequence homologous to *tms* but not *tmr*. Their results also suggested that Ri T-DNA may be discontinuous, analogous to the case with octopine T-DNA.

It has been shown that a portion of the Ti (Chilton, M.-D. *et al.* [1977] *Cell* 11:263-271) or Ri (Chilton, M.-D. [1982] *Nature* 295:432-434; White, F.F. *et al.* [1982]

de Framond, A.J. *et al.* (1983) *Biotechnol.* 1:262-269, have reported on the construction of a "mini-Ti plasmid." In the nopaline T-DNA there is normally only one site cut by the restriction enzyme *Kpn*I. A mutant lacking the site was constructed and a *Kpn*I fragment, containing the entire nopaline T-DNA, was isolated. This fragment together with a kanamycin resistance gene was inserted into pRK290, thereby resulting in a plasmid which could be maintained in *A. tumefaciens* and lacked almost all non-T-DNA Ti sequences. By itself, this plasmid was not able to transform plant cells. However, when placed in an *A. tumefaciens* strain containing an octopine Ti plasmid, tumors were induced which synthesized both octopine and nopaline. The mini-Ti plasmid has also been transferred into plant cells when complemented with a Ti plasmid deleted for its own T-DNA. These results indicated that the non-T-DNA functions acted in *trans* with T-DNA, that the missing nopaline Ti plasmid functions were complemented by the octopine Ti plasmid, and that the nopaline "mini-Ti" was functional in the transformation of plant cells. A similar pair of complementing plasmids, each containing either octopine T-DNA or *onc* genes, has been constructed by Hoekema, A. *et al.* (1983) *Nature* 303:179-180.

Chilton *et al.* (18 January 1983) 15th Miami Winter Symp., also reported on the construction of a "micro-Ti" plasmid made by resectioning the mini-Ti with *Sma*I to delete essentially all of T-DNA but the nopaline synthase gene and the left and right borders. The micro-Ti was inserted into a modified pRK290 plasmid that was missing its *Sma*I site, and was employed in a manner similar to mini-Ti, with comparable results.

Brief Summary of the Invention

One object of this invention is to confer pest resistance, specifically insect resistance, to a plant. In pursuance of this goal, other objects are to stably insert a gene coding for an insecticidal protein into the genome of the plant cell, to have this gene expressed in plant tissues, for the expression to be either regulated or constitutive, and for the plant tissues to be in a normal plant. Another object is to

unique pH400 *Bgl*II site being located between the *kan* gene and the *ocs* gene. The single *Bgl*II site is a convenient place to insert DNA, in particular if that DNA has compatible 5'GATC . . . 3' sticky-ends resulting from the action of *Bgl*II, *Bcl*I, or *Bam*HI.

12.2 Preparation of pDOB513. pDOB512, carrying cauliflower mosaic virus (CaMV) transcription controlling sequences (obtained from Dr. Ken Richards, Centre National de la Recherche Scientifique, Institute de Biologie Moleculaire et Cellulaire, 15, rue Descartes, F-67084 Strasbourg, France) was constructed as follows: A *Hin*dIII fragment carrying the CaMV 19S RNA promoter region (CaMV nucleotides 5376-5851) was inserted into pBR322 and was trimmed back to within one base pair of the 19S transcript cap site. An adapter molecule having both a *Sma*I site and a *Bam*HI site (the structure being 5'-CCCGGGGATC CGG-3':5'-CCGGATCCCC GGG-3', see below) was then ligated to the trimmed DNA. A *Hin*cII fragment carrying the CaMV 19S transcript terminator (CaMV nucleotides 7018-7794) to which *Bam*HI linkers had been added was then inserted behind the 19S promoter, the promoter and terminator being separated by the *Sma*I/*Bam*HI linker. The resulting plasmid is designated pDOB412. pDOB412 DNA was digested with *Bgl*II and *Sal*I, filled in by incubation with the Klenow fragment of *E. coli* DNA polymerase I, and religated, thereby deleting DNA, which includes *Bam*HI and *Hin*dIII sites, between the CaMV position 7644 *Bgl*II site and the pBR322 position 650 *Sal*I site and regenerating a *Bgl*II site. The resultant plasmid was designated pDOB512.

The sticky-ends of *Hin*dIII-linearized pDOB512 DNA were converted to blunt-ends. The blunt-ended pDOB512 DNA was mixed with and ligated to commercially available *Bgl*II linkers. The ligation mix was transformed into *E. coli* K802 and an ampicillin-resistant transformant was isolated which harbored a plasmid, designated pDOB513 (Figure 3). pDOB513 has CaMV 19S transcription controlling sequences on a *Bgl*II fragment. *Sma*I and *Bam*HI sites are found between the DNA segments having the promoter and the polyadenylation site in both pDOB412, pDOB512, and

pDOB513 DNA was digested with *Bgl*II, religated to itself, and transformed into K802. Colonies which harbored a plasmid, designated pDOB514, deleted for CaMV transcription controlling sequences were identified by restriction mapping of the harbored plasmids.

pCG116 DNA, which had, on a 2.26 kbp *Bgl*II fragment, a *Bam*HI site between the ORF24 promoter and transcript terminator, was then digested with *Bgl*II, which cleaves at sites which in T-DNA correspond to positions 18,027 and 21,522 *Eco*RI sites. The restriction digested DNA was mixed with and ligated to *Bgl*II-linearized pDOB514 DNA. Plasmid DNAs of ampicillin-resistant transformants were characterized by restriction mapping, and a colony was identified which harbored a plasmid, designated pMAN514, having a 2.29 kbp *Bgl*II fragment carrying an ORF24 promoter and polyadenylation site separated by a *Bam*HI site.

12.4 Preparation of the insecticide gene. After pBt73-16 was digested with *Nde*I, resulting *Nde*I sticky-ends were filled in by incubating the mixture with T4 DNA polymerase and all four dNTPs. The blunt-ended *Bacillus* DNA was mixed with and ligated to double-stranded, *Sma*I-linearized, M13mp19 RF DNA (Norrander, J. *et al.* [1983] *Gene* 26:101-106). The ligation mixture was transformed into *E. coli* JM105. DNAs isolated from plaques that were "clear" when plated on indicator plates were characterized by restriction analysis and a plaque was identified which harbored a vector, designated 1.6.4, having a 3.6 kbp *Bacillus thuringiensis* DNA oriented such that single-stranded form was complementary to crystal protein mRNA (i.e., the phage carried the antisense strand).

A *Bam*HI site was introduced into the *Bacillus* DNA immediately 5′ to the crystal protein translational start site essentially as described in Example 10. Sequences of the unmutated *Bacillus* DNA and the oligonucleotide primer are as follows:

```
                                TAA
Bacillus:  5'...GAGATGGAGG   CTTATGGATAAC...3'
Primer:       5'GAGATGGAGGATCCTTATGGATAAC3'
                          BamHI   MetTyrHis
```

12.7 Plant transformation. pH450 was transferred into *A. tumefaciens* LBA4404 (Ooms, G., *et al.* [1981] *Gene* 14:33-50), a *vir* gene-bearing micro-Ti-mobilizing strain, by the triparental mating technique. Tobacco leaf tissues were inoculated using a modification of the method of Horsch, R.B. *et al.* (1985) *Science* 227:1229-1231. Leaf segments were dipped in the inoculating suspension of bacteria for 1-3 minutes. The inoculating bacterial suspensions had titres of 10^7-10^8 ml^{-1}; the exact concentration was found to be unimportant. The medium was a tobacco regenerating medium having MS salts, a mixture well known to the art, supplemented with 0.1 mg/l p-chlorophenoxyacetic acid (pCPA), 7.5 mg/l 6-(2,2-dimethylallylamino)-purine (2iP), 300 mg/l kanamycin, and 125 mg/l cloxacillin, mefoxin and 500 mg/l carbenicillin. Generally 75-80% of the shoots were able to root in a kanamycin-containing medium and all were transformed. Some kanamycin-resistant shoots did not make measurable quantities of octopine.

12.8 Expression in plant tissues: immunoassays. Micro-ELISA double antibody sandwich assays were performed using a modification of the method of Example 7.

Antibodies were prepared by well-known methods. Coating antibody was partially purified mouse polyclonal antisera which was raised to *B. thuringiensis* HD-73 crystal protein. The mouse antisera was partially purified by precipitation with 50% (w/v) saturated ammonium sulfate at pH 7.4. The milky precipitate that forms during 30 minutes on ice was pelleted by centrifugation (20 minutes, 10,000 rpm). The pellet was then resuspended in phosphate buffered saline (PBS, pH 7.4) containing 137 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, and 8 mM $NaHPO_4$. The resulting coating antibody sample was then dialyzed overnight in PBS at 4°C.

Primary antibody was partially purified rabbit polyclonal antisera raised to *B. thuringiensis* HD-73 crystal protein. This antisera was cleared of antibodies that react with tobacco leaf protein. Tobacco leaf protein was bound to CNBr-activated "SEPHAROSE 4B" (Pharmacia, Upsalla, Sweden) (10 mg protein/ml gel). Rabbit serum was diluted 1:4 (v/v) with PBS and added to the swollen "SEPHAROSE" gel

A 1:2500 dilution of labeled secondary antibody (goat anti-rabbit IgG (H+L)-alkaline phosphatase-labeled) was then loaded into wells (100 μl/well). Plates were incubated for 1-2 hours at room temperature and thoroughly washed. Plates were washed 4-5 times with wash buffer. Plates were filled with wash buffer and placed on a shaker for at least 10 minutes. Plates were rinsed again and blotted dry.

An NADH substrate was then employed to detect bound labeled antibody. NADP (β-nicotinamide adenine dinucleotide phosphate) 0.25 mg/ml in 0.50 mM $MgCl_2$, 0.05 mM diethanolamine (pH 9.5) was loaded into the assay wells (100 μl/well). Plates were covered and stored at 4°C for about 1-1/2 hours.

Reagent solutions was prepared by dissolving p-Iodonitrotetrazolium violet (0.31 mg/ml) in 0.05 M sodium phosphate buffer (pH 7.2) containing 1.9% (v/v) ethanol. To this solution "DIAPHORASE" (NADH/dye oxidoreductase; Boehringer Mannheim, Mannheim, West Germany) to 0.035 mg/ml and alcohol dehydrogenase (baker's yeast) to 0.035 mg/ml were added. The reagent solution was incubated at 4°C for 45 minutes, after which it was added to the NADP solution in each assay well (150 μl/well).

The color reaction (red) should begin immediately. Optical density measurements were read at 492 nm.

Adjusted ELISA values of crystal protein in leaf tissue, expressed as absorbance at 492 nm, are reported in Table 7. The values were adjusted by subtracting from each measurement the value obtained for a *Nicotiana tabacum* var. "Xanthi" control. Many plants were observed to contain measurable amounts of antigen which bound anti-crystal protein antibody.

12.9 Expression in plant tissues: bioassays. Tobacco tissue transformed by pH450 was cloned and maintained in a growth chamber. For bioassay, leaves were cut off with a razor blade and placed in a 9 cm Petri dish lined with moist filter paper. Newly-hatched tobacco hornworm larvae (*Manduca sexta*) were put on the leaves. Mortality was recorded.

plasmid, p405/44-7, was digested with *Bgl*II and religated, thereby removing *Bacillus* sequences flanking the 3'-end of the crystal protein gene. The resulting plasmid, p405/54-12, was digested with *Pst*I and religated, thereby removing *Bacillus* sequences flanking the 5'-end of the crystal protein and about 150 bp from the 5'-end of the crystal protein structural gene. The resulting plasmid, p405/81-4, was digested with *Sph*I and *Pst*I and was mixed with and ligated to a synthetic linker having the following structure:

```
          SD        MetThrAla
 5'CAGGATCCAACAATGACTGCA3'
3'GTACGTCCTAGGTTGTTACTG5'
  SphI                 PstI
```

(SD indicates the location of a Shine-Dalgarno prokaryotic ribosome binding site.) The resulting plasmid, p544Pst-Met5, contains a structural gene encoding a protein identical to one encoded by pNSBP544 except for a deletion of the amino-terminal 47 amino acid residues. The protein encoded by p544Pst-Met5 is 2 amino acids longer than the 65 kDa toxic polypeptide processed from the 75 kDa crystal protein encoded by pNSBP544 (data not shown; see also McPherson *et al.* [1988] *Biotechnol.* 6:61-66). In bioassays, the proteins encoded by pNSBP544 and p544Pst-Met5 were shown to be equally toxic. All of the plasmids mentioned above have their crystal protein genes in the same orientation as the *lac*Z gene of the vector.

13.3 Modification of an insecticide gene's 3'-end. A *Hin*dIII site and a *Xma*I site were removed from the kanamycin resistance gene (*kan*), which encodes neomycin phosphotransferase I (NPTI), of pUC4K (Viera and Messing [1982] *Gene* 19:259-268) by the method of Merlo and Thompson (1987) *Anal. Biochem.* 163:79-87. The *kan* gene was removed from the resulting plasmid on a *Hin*cII fragment, and the ends were filled in by T4 DNA polymerase to make sure that they were blunt. pIC-20R (Marsh *et al.*, *supra*) DNA was digested with *Nde*I and *Sca*I and the ends were filled in by T4 polymerase. The resulting DNA, lacking the 5'-end of the ampicillin-resistance gene (*amp*) was mixed with and ligated to the NPT1-encoding *Hin*cII

and *B. thuringiensis* sequences was sequenced to confirm conservation of reading frame.

13.5 Construction of transcription vectors. An 833 base pair (bp) fragment was cut out of the pUC13 clone carrying the "−343" deletion (Odell *et al.* [1985] *Nature* 313:810-812) by digestion with *Sma*I and *Hin*dIII. This fragment carries a functional 35S promoter and the 5′-end of the CaMV 35S transcript, spanning from position −343 to position +9 relative to the transcriptional start site. This fragment was ligated into pIC19R (Marsh *et al.* [1984] *Gene* 32:481-485) which had been digested with *Nru*I and *Hin*dIII. The ORF25 polyadenylation site was contributed by a pTi15955 fragment spanning *Hin*cII sites at positions 21,727 and 22,440, as disclosed by Barker *et al.* (1983) *Plant Mol. Biol.* 2:335-350, ligated into the *Sma*I site of pIC19H (Marsh *et al.*, *supra*), the ORF25 polyadenylation site being proximal to the *Bam*HI site in the pIC19H polylinker. The T-DNA was then removed from the pIC19H vector on a *Bam*HI/*Bgl*II fragment which was then inserted into the *Bam*HI site of the pIC19R/35S promoter combination, the T-DNA being oriented so that the ORF25 polyadenylation site was proximal to the 35S promoter, a functional *Bam*HI site was between the CaMV and T-DNA sequences, and a *Bam*HI/*Bgl*II fusion was between the T-DNA and pIC19R sequences. This plasmid was then opened at the *Sma*I site between the fused *Bam*HI/*Bgl*II site and the pIC19R vector sequences. Plural *Bgl*II linkers were ligated into the *Sma*I site, resulting in formation of a *Pst*I site between the linkers. The resulting plasmid was designated pIC35/A.

A DNA linker encoding the tobacco mosaic virus (TMV) 5′-leader sequence was synthesized. The linker had the structure of

```
   (BglII)                                  Tth111II
5' GATCTATTTTTACAACAATTACCAACAACAACAAACAA...
3'     ATAAAAATGTTGTTAATGGTTGTTGTTGTTTGTT...

                                                    (BamHI)
                  ...CAAAACAACATTACAATTACTATTTACAATTACG     3'
                  ...GTTTTGTTGTAATGTTAATGATAAATGTTAATGCCTAG 5',
```

the horizontal lines indicating the location of sites recognized or partially recognized (partial recognition is indicated by parentheses) by the indicated restriction enzymes. This linker was inserted into pIC35/A's *Bam*HI site. The resulting plasmid, designated pIC35/A-TL4, had a *Bam*HI site between the TMV leader and the ORF25 polyadenylation site.

An NPT2 expression vector was constructed having the same CaMV DNA fragment carrying the 35S promoter as pIC35, the same NPT2 structural gene as used in Example 14.2.2 to construct p461/151-174, and the same T-DNA ORF25 polyadenylation site as used in pIC35/A. A 3.0 kbp *Hin*dIII fragment carrying the truncated Btt crystal protein gene of pNBSP544 was inserted into pIC19R-Kan or p35S-Kan.

13.6 Assembly of direct transformation vectors. The insecticide structural gene/fusion linker combination of p461/97-14 was removed by digestion with *Bgl*II and *Bam*HI and inserted into *Bam*HI-linearized pIC35/A-TL4 DNA. A plasmid, designated p461:151-193, was identified which had the insecticide structural gene oriented so that the 5′-end of the insecticide structural gene was proximal to the 35X promoter and the 3′-end of the linker was proximal to the ORF25 polyadenylation site.

The insecticide structural gene/fusion linker/NPT2 structural gene combination of p461/151-174 was removed by digestion with *Bgl*II and *Bam*HI and inserted into *Bam*HI-linearized pIC35/A-TL4 DNA. A plasmid, designated p461:162-191, was identified which had the insecticide structural gene oriented so that the 5′-end of the insecticide structural gene was proximal to the 35S promoter, and the 3′-end of the NPT2 structural gene was proximal to the ORF25 polyadenylation site.

13.7 Fusion of an insecticide gene with hygromycin sequences. An insecticide structural gene was derived from a 5.3-class gene (Kronstad and Whiteley [1986] *Gene* 43:29-40) from *B. thuringiensis* HD-1 (pBT1-106A, disclosed by Adang *et al.* [1987] in *Biotechnology in Invertebrate Pathology and Cell Culture* [K. Maramorosch,

(excised on a 1.45 kbp *Bam*HI fragment from pUC4K, Viera and Messing [1982] *Gene* 19:259-268), oriented to transcribe from left to right; a plant-expressible hygromycin-resistance gene, transcribed from right to left, under control of the CaMV 35S promoter and the T-DNA ORF25 polyadenylation site from pIC35/A, the hygromycin-resistant gene being derived from pLG62 (Gritz and Davies [1983] *Gene* 25:179-188); a dicistronic plant-expressible gene transcribed from right to left under control of the ORF24 promoter and either the ORF25 or ORF26 polyadenylation site (Barker *et al.*, *supra*), the dicistronic gene having an insecticide structural gene from HD-1 ("DIPEL") (a 4.5 kb-class gene, Kronstad and Whiteley, *supra*) at its 5'-end and a NPT2 structural gene (*kan*) at its 3' end; and a T-DNA fragment spanning positions 12,070 to 14,710, carrying an octopine synthase gene (*ocs*) transcribed to the left and the right T_L-DNA border repeat.

pH615 was identical to pH610 with some exceptions. There was no NPT1 gene. In the plant-expressible selectable marker, a Tn5 NPT2 structural gene (the "double mutant" of Sutton *et al.*, European Patent Publication No. 0 223 417) and an ORF26 polyadenylation site (from the same *Hin*cII fragment as contributed the ORF25 site) substituted from the hygromycin resistance structural gene and the ORF25 polyadenylation site of pH610. The plant-expressible insecticide gene was terminated by an ORF25 polyadenylation site. The insecticide structural gene was from HD-1 ("DIPEL"), and was preceded by a slightly modified alfalfa mosaic virus (AMV) RNA_4 5'-leader sequence. This leader was encoded by a synthetic linker having a structure of

```
(HindIII)                              HpaI          (NcoI)
5' AGCTTGTTTTTATTTTTAATTTTCTTTCAGTTAACTTCCAC      3'
3'      ACAAAAATAAAAATTAAAAGAAAGTCAATTGAAGGTGGTAC 5'
```

the horizontal lines indicating the location of sites recognized or partially recognized (partial recognition is indicated by parentheses) by the indicated restriction enzymes. This linker could be ligated to the *Hin*dIII site at the 3'-end of the 35S promoter, the resulting transcripts having 9 bp of the CaMV 35S transcript as its 5'-end. The *Nco*I

sticky-end could be ligated to the 5′-end of a structural gene by use of appropriately-tailed linkers.

pH619 was essentially identical to pH615 except for the insecticide structural gene and the polyadenylation site 3′ therefrom. The insecticidal structural gene of pH615 was the same as that carried by p544Pst-Met5, but lacking almost totally *Bacillus* sequences 3′ from the translational stop codon. The polyadenylation site 3′ from the structural gene was from T-DNA ORF25 and was carried, as described elsewhere herein, on a *Hin*cII fragment. Additionally, a TMV 5′-leader sequence (Example 13.5) was present between the 35S promoter and the p544Pst-Met5 structural gene.

pH623 was essentially identical to pH610 except for the presence of the TMV5′-leader (Example 13.5) at the 5′-end of the structural gene and for the substitution of the insecticide structural gene carried by p461/97-14 for the insecticide structural gene of pH610. The 3′-extension of the coding sequence beyond the natural position of the translational termination site did not affect toxicity of the encoded insecticidal protein.

pH624 was essentially identical to pH619 except for substitution of the insecticide/NPT2 structural gene of p461/151-174 for the insecticde structural gene of pH619.

pH627 was identical to pH615 with the exception of the presence of an inserted phaseolin third intron in the AMV RNA_4 5′-leader sequence. A fragment of a phaseolin gene, carrying the third intron and flanking coding sequences, and spanning from the *Xba*I site at position 904 to the *Sau*3AI site at position 1061 (as numbered by Slightom *et al.*, *supra*). This fragment could be inserted into the *Hpa*I site of the AMV RNA_4 leader linker with the aid of appropriate linkers to adapt *Xba*I and *Sau*3A sticky-ends to the blunt ends of *Hpa*I.

13.8 Deposited strains. The following strains were deposited with the Patent Culture Collection, Northern Regional Research Center, 1815 N. University Street, Peoria, Illinois 61604:

Strain	Deposit Date	Accession #
E. coli MC1061 (p544-HindIII)	10/06/87	NRRL B-18257
E. coli MC1061 (p544Pst-Met5)	10/06/87	NRRL B-18258

The deposited strains are provided for the convenience of those in the art, and are not necessary to practice the present invention, which may be practiced with the present disclosure in combination with publicly available protocols, information, and materials. *E. coli* MC1061, a good host for plasmid transformations, was disclosed by Casadaban and Cohen (1980) *J. Mol. Biol.* 138:179-207.

Example 14

This Example teaches expression in *Zea mays* of a coleopteran-active insecticidal protein gene from *Bacillus thuringiensis* var. *tenebrionis*.

14.1 Maize protoplast transformation. A suspension culture of Black Mexican Sweet (BMS) corn cells was digested in 2% cellulase, 0.25% pectinase (both from Worthington Biochemical Corp., Freehold, NJ), 0.2 M mannitol, and 80 mM $CaCl_2$ for about 4 hours at a concentration of about 1 g fr. wt. cells in 10 ml enzyme solution. The protoplast solution was filtered through a 46 μ mesh sieve to remove undigested cell clumps. Protoplasts were obtained from 8.5 g fr. wt. of cells. After washing, the protoplasts were resuspended in MaMg (0.4 M mannitol, 15 mM $MgCl_2$, 0.1% MES, pH 5.6) at a concentration of 2.5 x 10^6 ml^{-1}. Half ml aliquots of protoplasts were placed into 15 ml disposable round-bottom centrifuge tubes. Appropriate DNA solutions were added to each tube, followed by the addition of 300 μl of a 40% (w/v) polyethylene glycol (PEG) solution (avg. PEG molecular weight = 6000-7500; source: EM Science, Gibbstown, NJ; dissolved in NaMg; final pH about 6.0; filter-sterilized). DNA solutions were as follows:

	BTT	Fusion-High	Fusion-Low	Control
μg p461:162-191 DNA	--	10	2.5	--
μg p461:151-193 DNA	25	--	--	--
μg pIC19R-35S-Kan DNA	2.5	--	--	--
μg salmon sperm carrier DNA	22.5	40	47.5	50
Total DNA conc. (μg/ml)	100	100	100	100

The tubes were incubated for 30 minutes at room temperature with occasional gentle mixing. The incubations were then diluted by addition of and mixing with 1 ml of MS4D+8M (MS salts, 4 mg/l 2,4-D, 8% mannitol, 2% sucrose, 0.75 mg/l thiamine·HCl, 7.7 mg/l glycine, 1.3 mg/l nicotinic acid, 0.25 mg/l pyridoxine·HCl, 0.25 mg/l calcium pantothenate, and 1 mM asparagine). After a further 5 minutes, two further dilutions of 2 ml MS4D+8M were done at 5 minute intervals. The protoplasts were centrifuged at low speed, resuspended at a concentration of 2×10^5 m^{-1} in CM + 8% mannitol (CM = conditioned medium = filter-sterilized medium that BMS suspension cells had been growing in), poured into a 100 x 20 mm Petri plate, diluted with an equal volume of MS4D+8M, 2.4% SP (Sea Plaque agarose, FMC BioProducts, Rockland, ME) at about 37°C, and swirled to evenly disperse protoplasts. After the medium had solidified, the Petri plates were sealed with parafilm, placed in plastic storage boxes, and incubated in very dim light at about 26°C.

After 12 days, 12.5 ml of MS4D + 4% mannitol + 100 mg/l kanamycin was added to the plates, resulting in a final selective kanamycin concentration of about 50 mg/l. Nine days later, agarose slabs containing developing protoplast-derived colonies were replated onto Gel-rite-solidified MS4D + 100 mg/l kanamycin in 100 x 15 mm Petri plates. Kanamycin-resistant calli developed within 3 weeks from cell treatments except the "Control." The kanamycin-resistant calli were transferred individually to fresh Gel-rite solidified MS4D + 100 mg/l kanamycin, and were

subsequently maintained by transferring every 2 to 3 weeks onto medium of the same composition.

After selection on kanamycin, no "Control" calli remained, and three "Fusion-Low" calli, seven "Fusion-High" calli, and 169 "BTT" calli had survived.

14.2 Assay of insecticidal protein. Presence of the introduced DNA sequences was analyzed by Southern blot hybridization, using nick-translated NPT2 and insecticide gene DNA fragments (IG) as probes. Expression of the introduced genes was assayed by ELISA, using anti-NPT2 and anti-*B. thuringiensis* var. *tenebrionis* crystal protein antisera (CP). Separate tissue samples from each cell line were used for each assay. The results of the ELISA assays and Southern blots on the "Fusion" calli are summarized in Table 10.

ELISA positive were obtained only from calli whose DNA had regions of homology to both the NPT2 and IG probes. The lack of complete correspondence between the NPT2 and CP ELISAs could be due to reasons such as differential sensitivity of the assays, occasional problems with protein extraction or protein stability, or chimeric cell lines.

Twenty-eight of the 169 "BTT" calli were assayed by ELISA on two different days (separate tissue samples), using the anti-insecticidal protein antiserum. The results are shown in Table 11. Nine out of 28 (32%) of the calli tested positive in at least one of the two assays. There were only three discrepancies between the two assays (calli 6-21, 6-32, and 21-12). In each case, the callus tested positive in Experiment 1 and negative in Experiment 2. The most likely explanation is lower sensitivity of the Experiment 1 assay compared to the Experiment 2 assay.

Example 15

This Example teaches the expression in tomato (*Lycopersicum esculentum*) of the full-length *Bacillus thuringiensis* insecticide gene under control of both viral and T-DNA promoters.

15.1 Leaf tissue transformation. The leaf tissue transformation protocol combined and modified the procedures of Bevan, M. *et al.* (1985) *EMBO J.* 4:1921-1926, and Horsch, R.F. *et al.* (1985) *Science* 227:1229-1231. Leaves from three- to five-week-old greenhouse-grown seedlings were used. These leaves were washed, surface-sterilized, brushed, cut into 5 mm sections, and placed on agar-solidified regeneration medium (MS salts [Murashige, T., F. Skoog (1962) *Physiol. Plant.* 15:473-497], 30 g/l sucrose, 100 mg/l thiamine, 50 mg/l nicotinic acid, 50 mg/l pyridoxine, 5 μM zeatin riboside, 3 μM 3-indole-acetyl-DL-aspartic acid, 9 g/l agar, pH 5.65-5.8) or moistened filter paper for 1 day before bacterial inoculation. It was important to keep leaves moist. There were noticeable increases in survival with the 24-hour culture period prior to bacterial inoculation. Overnight cultures of bacteria were grown in L broth on selective antibiotics and diluted about 1/10 (an OD at 595 nm of about 250) prior to use. Bacteria were applied to each leaf disc with a sterile paintbrush and then plates were placed at 25°C for 2-3 days. After the 2 days, leaf discs were rinsed in antibiotic-containing regeneration media and then placed on antibiotic-containing regeneration agar plates. (All plates had an antibiotic-containing medium, containing 50 mg/l cefotoxin, or 250 mg/l carbenicillin and 50 mg/l vancomycin.)

Leaves transformed by bacterial strains containing the kanamycin resistance genes were grown under selection pressure using three procedures (Colbere-Garapin, F. *et al.* [1981] *J. Mol. Biol.* 15:1-14): (1) placement of inoculated explant on antibiotic-containing medium with 20 mg/l G418; (2) placement of explants on antibiotic medium with 5 mg/l G418; and (3) placement of explants on antibiotic medium with no G418. All treatments were transferred to plates containing about 15 to 20 mg/l G418 after approximately 5-7 days. An additional transfer two weeks later helped to eliminate further escapes. Tissues were then tested for opines.

15.2 Hypocotyl transformation. The use of inverted stem segments for *in vitro* transformation of tobacco tissues was first developed by Braun, A.C. (1956) *Cancer Res.* 16:53-56. The key to success using this method appears to be the fact that

wound cell divisions and callus formation occur at the basal end of the stem or hypocotyl segment. These divisions are important for two reasons. First, wound responses are a known requirement for transformation by *Agrobacterium tumefaciens*. Second, and more important, is that the subsequent cell divisions increase the numbers of transformed cells to a point where they can be screened for by opine analysis and survive heavy kanamycin selection pressure. The standard protocol used was to isolate hypocotyl segments (from sterile plants grown in growth incubator), to invert these so that the basal end was up, and then to inoculate with 2 μl bacteria applied with a micropipet (Pipetman). Bacteria were grown in L broth of MSSP medium supplemented with 50 μM acetosyrigone (Stachel, S.E., *et al.* [1985] *Nature* 318:624-629). After 1 to 2 days, the segment was washed thoroughly with liquid medium containing antibiotic and transferred to solidified antibiotic-containing medium. After visible callus was observed, it was excised, and cut into small pieces (about 3 mm) and subcultured on agar-solidified medium containing antibiotics. (These antibiotics included carbenicillin 260 mg/l and vancomycin 50 mg/l and G418 for selection pressure.) After tissues were grown to a large enough size (about 50 to 100 mg), they were subcultured and analyzed for opine content.

15.3 Transformations with *Agrobacterium* strains. Strains used for transformation of tomato were *A. tumefaciens* LBA4404 (Ooms, G. *et al.* [1981] *Gene* 41:33-50) containing the binary vectors pH450, pH575, pH576, pH578, pH582, and pH585. Transformations were made as described above on both leaf and hypocotyl tissues of UC82, LA14, V7 (LA14 X LA159) and V7R (LA159 X LA14) hybrids (carrying several marked genes in a heterozygous state), and proprietary hybrids 6-16, 6t0016, and 6t0045 M. Hypocotyl transformations generally yielded higher efficiency of transformation as detected by octopine production and kanamycin resistance. Efficiency of leaf transformations varied in the range of about 0.5% to about 5.0% depending on the particular *Agrobacterium* strains while hypocotyl transformations ranged in efficiency from about 5 to about 40% (Table 12).

Tomato plants were regenerated as described by Tatchell, S., A. Bins (1986) *Tomato Genet. Coop. Rept.* No. 36, pp. 35-36.

15.4 ELISAs. ELISA protocols were based on those described in Example 12.8. Techniques for preparing tissue samples were modified to enhance detection of insecticidal protein in plant tissues. Since in plant extracts, solubilization can be incomplete, this centrifugation step was eliminated. Leaf tissue was harvested from greenhouse plants, weighed, and placed in Eppendorf tubes on ice. Samples were quickly frozen in liquid nitrogen, thawed briefly, ground with a glass rod, and resuspended in 5X W/V PBS with 0.1 mM phenylmethylsulfonyl fluoride (PMSF). These samples are placed in the cold for about twenty minutes before taking aliquots for protein assays. Modified double antibody sandwich ELISAs with NADP-enhanced alkaline phosphatase were performed, tissue extracts containing 0.2 to 0.5 mg/ml protein (Clark, M.F., M. Bar-Joseph [1984] *Meth. Virol.* 7:51-85). Polystyrene microtiter plates were coated with mouse polyclonal antisera in 15 mM sodium carbonate buffer, 35 mM sodium bicarbonate, and pH 9.6 (0.1 ml/well) and stored at 4°C for 1 day to 2 weeks. Between each step, plates were washed 3 times with PBS-Tween (PBS + 0.05% "TWEEN"). Plates were blocked with blocking solution (PBS + "TWEEN" + 1% bovine serum albumin (BSA) fraction V (Sigma) and 1% casein acid hydrolysate (Sigma)). Plates were washed again and 0.1 ml/well antigen solution was added and incubated for about 2 to 3 hours at 25°C. Primary rabbit antisera against *B. thuringiensis* insecticidal protein were added to washed plates and incubated overnight at 4°C. The following morning, plates were washed and a 1:2500 dilution of alkaline phosphatase-labeled goat anti-rabbit antibody (Kirkegaard Perry Laboratories, Inc.) was added for about 2 hours. Plates were developed with modified nicotinamide adenine dinucleotide phosphate (NADP) enhancement (Johansson, A. *et al.* [1986] *J. Immun. Meth.* 87:7-11; Stanley, C.J. *et al.* [1985] *J. Immun. Meth.* 83:89-95; Self, C.H. [1985] *J. Immun. Meth.* 76:389-393). This involved addition of 0.1 ml/well of 300 mM NADP in diethanolamine substrate buffer (50 mM diethanolamine HCl pH 9.5, 1 mM $MgCl_2$) followed 25 minutes later by addition of

amplifier buffer (0.05 M sodium phosphate buffer, pH 7.2, containing 5 mg/ml BSA and 4% ethanol) with 0.55 mM *p*-iodonitrotetrazolium (Sigma), 1.5 mg/ml "DIAPHORASE" (Boehringer Mannheim), and 2 mg/ml alcohol dehydrogenase (Sigma). The reaction was stopped by the addition of 0.2 M H_2SO_4. Absorbances were read at 492 nm. Alternate development used was conventional *p*-nitrophenyl phosphate development.

15.5 Western blots. *Bacillus thuringiensis* insecticidal protein was detected in transformed tomato plants using protein immunoblot procedures ("Westerns"). The basic protocol involved preparation of leaf tissue by freezing in liquid nitrogen, grinding with mortar and pestle, and precipitating protein with 10% trichloroacetic acid. The samples were incubated on ice for at least 30 minutes and then spun at approximately 9000 rpm for 10 minutes. The pellet was resuspended in phosphate-buffered saline (PBS: 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4), and then insecticidal protein was precipitated in 20% ammonium sulfate, saturated ammonium sulfate being added slowly to samples at 4°C. Samples were then spun for 10 minutes at 9000 rpm, pellets were resuspended in modified PBS (PBS, 1% polyvinyl pyrrolidone, 0.006% 2-mercaptoethanol (2ME), 0.01% PMSF) and then dialyzed in PBS overnight at 4°C. Samples were cleared through glass wool and frozen at −20°C in cracking buffer (5% SDS, 6 M urea, 20 mM NaH_2PO_4, 0.02% bromophenol blue, 20% glycerol, and 0.3% 2ME). Tissue samples were then loaded on 8.5% acrylamide stacking gel (Laemmli, U.K. [1970] *Nature* 227:680-685). The stacking gel was run at 60 volts for about 1 hour and the running gel at 120 volts for 2 hours. Proteins were transferred to nitrocellulose (BA85) in Towbin's buffer (Towbin, H. *et al.* [1979] *Proc. Natl. Acad. Sci. USA* 76:4350-4354) in an IDEA Scientific GENIE Blotter at about 1 A and about 24 V for about 1 to 2 hours at 4°C. The nitrocellulose was briefly fixed in Towbin's buffer and then blocked for 1 hour at room temperature in Tris-buffered saline (TBS: 50 mM Tris-HCl, 0.15 M NaCl, pH 7.5) with 5% nonfat, dry mild (NFDM; Carnation). After blocking, it was washed 3 times (20 minutes each) with TBS + 0.5% NFDM + 0.5% Nonidet P-40 (detergent) before incubating

overnight at 4°C with anti-insecticidal protein antibodies. After primary antibody infection, the nitrocellulose was again washed 3 times (20 minutes each) with TBS + 0.5% NFDM + 0.5% sodium dodecyl sulfate (SDS). It was then incubated with goat anti-rabbit antibody alkaline phosphatase labeled (1:4000) for 2 hours at room temperature. Again, the nitrocellulose was washed for 20 minutes in TBS + 0.5% NFDM + 0.5% SDS, followed by 2 additional washes (10 minutes each) with TBS + 0.5% NFDM + 0.05% "TWEEN." The nitrocellulose was rinsed quickly three times in $MgCl_2$ buffer (0.1 M Tris HCl, pH 8.8, 0.1 M NaCl, and 5 mM $MgCl_2$) and then developed with 0.15 mg/ml 5-bromo-4-chloroinoxylphosphate/nitroblue tetrazolium substrate (Knecht, D.A., R.L. Dimond [1984] *Anal. Biochem.* 136:180-184; Blake, M.S. *et al.* [1984] *Anal. Biochem.* 136:175-179). Extracts of control tissues were spiked with protoxin and/or toxin during extraction to estimate recovery during the Western procedure. This showed that often insecticidal protein did not completely solubilize. As little as 0.01 ng of toxin was routinely detected on the blot. In 'spiked' samples, about 10 ng of standard was added to typical UC82 extract (from 100 mg tissue); the resultant signal on the blot represented about 30% recovery.

15.6 Bioassays. Bioassays were the most sensitive assay for the detection of *Bacillus thuringiensis* insecticidal protein. Two types of bioassays were conducted on putative transgenic plants. In the first, leaves were excised and placed in Petri plates containing moistened filter paper. A predetermined number of first instar *Manduca sexta* (tobacco hornworm) larvae were then added. This assay facilitated observations on *Manduca* and enabled larger numbers of samples to be tested. Substantial differences were observed between some varieties. However, results on a given R_0 or R_1 plant were not always consistent, due to either instability or insecticidal protein in excised leaves, or possible feeding of worms on moistened filter paper. In the second type of bioassay, first instar or, preferably, neonatal hornworms just hatched from eggs (eggs from Carolina Biological Supply) were placed on plants in the greenhouse, and checked daily for growth and mortality after 7 to 9 days. Problems with this assay included worms leaving tomato plants and dying of desiccation due to

pH450, pH577, and pH578, and in R_1 plants of pH577 and pH582 transformations. All ELISA-positive plants tested killed some *Manduca sexta* larvae in bioassays (Table 13).

Western blots were used to determine the form of insecticidal protein expressed (protoxin or toxin) and to quantify the level of expression more precisely. In pH450 transformants, which contain the full-length *Bacillus thuringiensis* insecticidal protein structural gene, both protoxin and toxin were observed in leaf tissues. Tissues from pH577 and pH578 transformed plants also had both forms of the insecticidal protein. Different forms of insecticidal protein (protoxin and toxin) were also observed in different regenerated plants from what initially appeared to be a single transformation event.

Results for many of the plants assayed by bioassay are listed in Table 13. Further analysis (not shown) by analysis of variance (ANOV) and Duncan's multiple range test showed significant differences at the 1% level. Transformed plants from pH450 and pH577 gave the best biological activity. Decreases in biological activity of transformed plants as a function of plant and leaf age were also observed. Control plants (UC82 R_0 plants and UC82 plants grown from seed) all gave low rates of mortality (less than 25%). It was significant that on several of the R_1 plants with lower *Manduca* mortality, the worm weights were significantly lower than controls. In addition, *M. sexta* larvae that failed to die immediately on *B. thuringiensis* insecticidal protein-containing plants also failed to grow at normal growth rates. These differences were clearly apparent after 2 weeks.

Southern analysis showed that insertions of T-DNA containing the plant-expressible insecticide gene were present in the expected organization, and are usually present in low copy number (about 1 to 2 copies/haploid genome). In all plants positive for protein and in bioassay positive plants tested, insecticidal protein sequences were observed in Northern blots of polyadenylated RNA. However, most of this RNA was shorter than expected.

aspartic acid (Research Organics) and 1.76 mg/l zeatin riboside (Sigma). Hormones were added prior to autoclaving. The pH was adjusted to 5.8 with 0.1 M KOH and the medium was solidified with 7 g/l Tonomeko agar. Shoot induction medium containing carbenicillin at 500 mg/l and kanamycin at 25 mg/l was used for selection of transformed shoots. Stock plantlets were grown on MS basal medium without hormones in magenta GA7 containers (Magenta Corp.) and subcultured every 4 weeks. Shoot elongation and rooting medium consisted of MS basal medium with 25 mg/l kanamycin.

16.4 Procedure. Both stem and tuber tissue were used for transformation. Tubers were peeled and surface-sterilized in 10% liquid bleach for 15 minutes under constant agitation. A 0.7 cm diameter cork borer was used to make cores through the tuber. These cores were then sectioned into 2 to 3 mm thick discs and then inoculated (Sheerman, S., M.B. Bevan [1988] *Plant Cell Rep.* 7:13-16). Stem sections approximately 0.5 cm were made from *in vitro* grown stock plants 2 to 3 weeks after subculture. All sections containing axillary buds were discarded. Stem or tuber tissues were immediately soaked in 10^6, 10^7, or 10^8 ml^{-1} *Agrobacterium tumefaciens* cells for 5 to 10 minutes. They were then placed on sterile filter paper discs over *Nicotiana tabacum* feeder layers (Horsch, R.B. *et al.* [1985] *Science* 227:1229-1231) over shoot induction medium without antibiotics. They were cocultivated for 2 days then rinsed in liquid MS media containing 500 mg/l carbenicillin, blotted on sterile cardboard, and transferred to shoot induction media with carbenicillin 500 mg/l. After 4 to 5 days the stem segments or tuber discs were placed on shoot induction media containing 25 mg/l kanamycin and 500 mg/l carbenicillin. Cultures were transferred every 3 weeks. Buds began to appear in 4 to 6 weeks and continued to form for several weeks thereafter. Shoots were excised and rooted on MS media with 25 mg/l kanamycin, then tested for gene expression.

16.5 Results. Thirty-three Russet Burbank plants regenerated from tissues inoculated with *A. tumefaciens* (pH615) were selected on 25 mg/l kanamycin. Three of these plants were tested for expression of *B. thuringiensis* insecticidal protein by

C. tarsalis (western encephalitis mosquito)
C. territans
C. univittatus
Culiseta incidens (*Culiseta*: mosquitos)
C. inornata
Diamessa sp.
Dixa sp. (*Dixa*: midges)
Eusimulium (*Simulium*) *latipes* (*Eusimulium*: gnats)
Goeldichironomus holoprasinus
Haematobia irritans (horn fly)
Hippelates collusor
Odagmia ornata
Pales pavida
Polpomyia sp. (*Polpomyia*: midges, biting)
Polypedilum sp. (*Polypedilum*: midges)
Psorophora ciliata
P. columiae (*confinnis*) (Florida glades mosquito, dark rice field mosquito)
P. ferox
Simulium alcocki (*Simulium*: black flies)
S. argus
S. cervicornatum
S. damnosum
S. jenningsi
S. piperi
S. tescorum
S. tuberosum
S. unicornatum
S. venustum
S. verecundum
S. vittatum
Uranotaenia inguiculata
U. lowii
Wyeomyia mitchellii (*Wyeomyia*: mosquitos)
W. vanduzeei

HYMENOPTERA

Athalia rosae (as *colibri*)
Nematus (*Pteronidea*) *ribesii* (imported currantworm)
Neodiprion banksianae (jack-pine sawfly)
Priophorus tristis
Pristiphora erichsonii (larch sawfly)

LEPIDOPTERA

Achaea janata
Achroia grisella (lesser wax moth)
Achyra rantalis

Nymphalis antiopa (mourning-cloak butterfly)
Oiketicus moyanoi
Ommatopteryx texana
Operophtera brumata (winter moth)
Opsophanes sp.
O. fagata
Orgyia (*Hemerocampa*) *antiqua*
O. leucostigma (white-marked tussock moth)
O. (*H.*) *pseudotusgata* (Douglas-fir tussock moth)
O. thyellina
Orthosia gothica
Ostrinia (*Pyrausta*) *nubialis* (European corn borer)
Paleacrita vernata (spring cankerworm)
Pammene juliana
Pandemis dumetana
P. pyrusana
Panolis flammea
Papilio cresphontes (orangedog)
P. demoleus
P. philenor
Paralipsa (*Aphemia*) *gularis*
Paralobesia viteana
Paramyelosis transitella
Parnara guttata
Pectinophora gossypiella (pink bollworm)
Pericallia ricini
Peridroma saucia (variegated cutworm)
Phalera bucephala
Phlogophora meticulosa
Phryganidia californica (California oakworm)
Phthorimaea (= *Gnorimoschema*) *operculella* (potato tuberworms)
Phyllonorycter (*Lithocolletis*) *blancardella*
Pieris brassicae (large white butterfly)
P. canidia sordida
P. rapae (imported cabbageworm, small white butterfly)
Plathypena scabra (green cloverworm)
Platynota sp.
P. stultana
Platyptilia carduidactyla (artichoke plume moth)
Plodia interpunctella (Indian-meal moth)
Plutella xylostella as *maculipennis* (diamondback moth)
Prays citri (citrus flower moth)
P. oleae (olive moth)
Pseudoplusia includens (soybean looper)

Pygaera anastomosis
Rachiplusia ou
Rhyacionia buoliana (European pine shoot moth)
Sabulodes caberata
Samia cynthia
Saturnia pavonia
Schizura concinna (red-humped caterpillar)
Schoenobius bipunctifer
Selenephera lunigera
Sesamia inferens
Sibine apicalis
Sitotroga cerealella (Angoumois grain moth)
Sparganothis pilleriana
Spilonota (*Tmetocera*) *ocellana* (eye-spotted budmoth)
Spilosoma lubricipeda (as *menthastri*)
S. virginica
Spilosoma sp.
Spodoptera (*Prodenia*) *eridania* (southern armyworm)
S. exigua (beet armyworm, lucerne caterpillar)
S. frugiperda
S. littoralis
S. litura
S. mauritia
S. (*P.*) *ornithogalli* (yellow-striped armyworm)
S. (*P.*) *praefica*
Syllepte derogata
S. silicalis
Symmerista canicosta
Thaumetopoea pityocampa (pine processionary caterpillar)
T. processionea
T. wauaria (currant webworm)
T. wilkinsoni
Thymelicus lineola (European skipper)
Thyridopteryx ephemeraeformis (bagworm)
Tineola bisselliella (webbing clothes moth)
Tortrix viridana (oak tortricid)
Trichoplusia ni (cabbage looper)
Udea profundalis (celery leaf tier)
U. rubigalis
Vanessa cardui (painted-lady)
V. io
Xanthopastis timais
Xestia (*Amathes, Agrotis*) *c-nigrum* (spotted cutworm)
Yponomeuta cognatella (= *Y. evonymi*) (*Yponomeuta* = *Hyponomeuta*)

Table 4 (continued)

Strain or Plasmid	Constructed or used in Example	See Figure	Made From (α comments)
p403B/BTBΔ3	12.3	4	1.6.4B-3.8.3, p403B
pH4-1	12.1		pSUP106, pTi15955, CaMV, Tn*5*
pH400	12.1		pH4-1
pDOB412	12.2		CaMV, pBR322
pDOB512	12.2		pDOB412
p403BRL1	12.3		p403B
pDOB514	12.3		pDOB513
pMAN514	12.3		p403BRL1, pDOB514
pKS4.2	12.3		pKS4
pKS4.3	12.3		pKS4.2
pBR322Bam	12.3		pBR322, *lambda*
p11-83c	12.3		pKS4.3, pBR322Bam$^-$
pCJ161	12.5		1.6.4B-3.8.3, pCG116
pH450	12.6		pCJ161, pH400
pCG116	12.3		p11-83c, pTR-*pro*I(*Bam*)

Table 7.

	Experiment 1[5]		Experiment 2[6]	
Clone	A_{492}[1]	CP[2]	A_{492}	CP
100	0.17 ± 0.03[7]	+	0.14	+
101	0.02 ± 0.01	–	0.07	±
102	0.06 ± 0.02	±	0.21	+
103	0.00 ± 0.00	–	ND	ND
104	ND[3]	ND	0.11	+
105	0.00 ± 0.00	–	0.10	+
106	0.10 ± 0.02	+	0.01	–
107	0.06 ± 0.03	±	0.06	–
109	0.11 ± 0.05	+	0.03	–
110	0.06 ± 0.01	±	0.13	+
111	0.12 ± 0.02	+	0.00	–
NX[4]	-0-		-0-	

[1]Average of three ELISA determinations. Absorbance at 492 nm, corrected by subtracting the value for the NX control.

[2]Rated as having (+) or not having (–) crystal protein. ± indicates a marginal rating.

[3]Not determined.

[4]Untransformed *Nicotiana tabacum* var. "Xanthi" control. In experiments 1 and 2, repectively, 18 and 19 control leaves obtained from different plants were averaged.

[5]Experiment 1 was standardized for equal tissue wet weight.

[6]Experiment 2 was standardized for equal plant protein concentrations.

[7]± standard error of the mean.

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on 5 JANUARY 2006.

#35,589

Jeff Lloyd, Patent Attorney

REQUEST FOR RECONSIDERATION
UNDER 37 CFR 1.111
Patent Application
Examining Group 1638
Docket No. MPS 11-83D2D1
Serial No. 08/478,153
Confirmation No. 6814

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	David T. Fox
Art Unit	:	1638
Applicants	:	Michael J. Adang, John D. Kemp
Serial No.	:	08/478,153
Conf. No.	:	6814
Filed	:	June 7, 1995
For	:	Insect Resistant Plants

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR RECONSIDERATION UNDER 37 CFR 1.111

Sir:

In response to the Communication mailed August 27, 2002, regarding suspension of prosecution in the application identified above, Applicants respectfully request reconsideration of the decision to suspend prosecution in view of the accompanying amendments and remarks.

Please amend the application identified above as follows.

In the Claims

Please cancel claims 20-23, without prejudice.

Please add the following new claims:

24. (New) A regenerated cotton plant selected from the group consisting of (1) a plant of a Class 2 *Gossypium* genotype transformed to contain selected foreign DNA and having a phenotype conferred by said foreign DNA by which said cotton plant can be distinguished from naturally-occurring cotton plants, and (2) descendants of said cotton plant having said distinguishing phenotype.

25. (New) A transgenic cotton plant according to claim 24 comprising an insecticide gene under control of a plant-expressible promoter wherein said insecticide structural gene encodes the amino acid sequence of Figure 1 (SEQ ID NO:2) and is expressed under control of said promoter such that tissues of said plant are toxic to insects.

26. (New) A process for regenerating a whole plant of the genus *Gossypium* comprising:

(a) culturing tissue from a plant of said genus on a callus initiation medium having a high cytokinin/auxin ratio to proliferate callus;

(b) culturing the callus of step (a) on a somatic embryo induction medium having a high auxin/cytokinin ratio to produce embryogenic calli;

(c) culturing the embryogenic calli of step (b) on suitable media for production of somatic embryos, embryo maturation, embryo germination and plant regeneration.

27. (New) The process of claim 26 in which the plant is a Class 2 cultivar.

28. (New) The process of claim 26 in which the auxin is NAA.

29. (New) The process of claim 26 in which the cytokinin is 2iP.

30. (New) The process of claim 26 in which the somatic embryo production medium of step (c) is a phytohormone-free medium.

31. (New) The process of claim 26 in which said tissue is taken from seedlings.

32. (New) The process of claim 31 in which said tissue comprises cotyledon tissue.

33. (New) The process of claim 31 in which said tissue comprises hypocotyl tissue.

34. (New) The process of claim 26 in which said tissue is taken from immature embryos.

35. (New) The process of claim 26 in which the embryo maturation medium of step (c) contains no phytohormones.

36. (New) The process of claim 26 in which the embryo maturation medium of step (c) contains zeatin, NAA and a gibberellin.

37. (New) The process of claim 26 in which the embryo germination medium of step (c) comprises GRM_{gn}.

38. (New) The process of claim 26 in which the plant regeneration medium of step (c) comprises 2 G_0.

39. (New) The process of claim 26 in which the plant regeneration medium of step (c) comprises GRM_{gn}.

40. (New) The process of claim 26 in which said tissue is transformed to contain selected foreign DNA.

41. (New) The process of claim 40 in which said tissue is transformed by contacting it with *Agrobacterium* containing said foreign DNA.

42. (New) The process of claim 40 in which a whole plant containing foreign DNA is regenerated.

43. (New) The process of claim 40 in which said plant expresses said foreign DNA.

44. (New) A plant produced by the method of claim 41 having a phenotype conferred by said foreign DNA by which said plant can be distinguished from a naturally-occurring plant.

45. (New) A seed of a plant of claim 44.

46. (New) In a method for regenerating a cotton (*Gossypium*) plant comprising culturing somatic tissue thereof on suitable media to cause callus formation and whole plant regeneration, the improvement comprising using somatic tissue of a Class 2 genotype of a *Gossypium* species and culturing on a callus initiation medium having a high cytokinin/auxin ratio followed by culturing on an embryo induction medium having a high auxin/cytokinin ratio.

In the Specification

On page 25, line 6, above "Detailed Description of the Invention" please insert the following paragraph:

--**Figure 4** is a diagram illustrating a regeneration scheme for cotton of this invention. The abbreviations G0 through G3, $MS_{zn-g,}$ 1/2 G0 and GRM_{gn} are described in Table 14 hereof.--

Please substitute the following paragraphs:

Page 98, line 21 through page 99, line 3:

Cotton was transformed essentially as disclosed by Firoozabody, E. *et al.* (1987) *Plant Mol. Biol.* 10:105-116, and Firoozabady, E. U.S. Patent Application Serial No. 07/076,339. The Firoozabady application reads, in pertinent part: Cotton (genus *Gossypium*) is an important commercial crop. Fiber-producing members of this genus are *G. arboreum*, *G. herbaceum*, *G. hirsutum*, *G. barbadense*, *G. lanceolatum*, all the foregoing being cultivated species, and *G. tomentosum*, *G. mustelinum* and *G. darwini* which are wild-type species. "Cotton," R.J. Kohel *et al.* eds. (1984), American Society of Agronomy, Inc., p. 52. In the United States, *G. hirsutum* is the major cultivated species. A number of different varieties are cultivated in different parts of the country, classified into Acala, Delta, Plains and Eastern. The Acala varieties grown in the Southwest are predominantly Acala 17's, and in California are the SJ series. Delta varieties include Stoneville and Deltapine. Plains varieties include Lankart and Paymaster, and Eastern varieties include Coker and McNair. Cotton, *supra*, p. 203-205. Southwestern varieties also include the GSA varieties.

Despite success in regenerating a number of plants such as tobacco and petunia, investigators have had substantial difficulties with regeneration of cotton (*Gossypium hirsutum L.*). Methods of regenerating this valuable crop plant from somatic tissue are desirable so as to enable transformation of cotton with foreign DNA conveying valuable agronomic traits. Limited success has been obtained in the regeneration of easily regenerable Class 1 genotypes of cotton such as the Coker varieties grown primarily in the eastern United States, but to date no methods have been available for the regeneration and transformation of Class 2 agronomic genotypes such as those of the Acala, Delta and Plains types which make up the important crop varieties of the remainder of the United States. The methods by which this invention achieves regeneration involve adjustments of auxin/cytokinin

ratios in somatic embryogenesis induction media. No prior disclosures teach or suggest such adjustments for the regeneration of any variety of cotton.

A practical, reproducible, efficient method for regenerating fiber-producing species of cotton as provided herein is useful in rapid multiplication of plants produced by conventional breeding methods and in genetic engineering of plants wherein foreign genes are introduced into plant cells and the cells are regenerated to form whole fertile plants.

Ammirato, P.V. (1983) "Embryogenesis," in Handbook of Plant Cell Culture, Evans, D.A., *et al.*, eds. *1*:82-123 provides a general discussion of somatic embryogenesis as a method of plant regeneration. This article at page 84 describes the basic somatic embryogenesis protocol as involving a primary medium with an auxin source and a second medium devoid of growth regulators. During the primary culture the tissue underwent differentiation to produce a mass of unorganized cells and cell clusters, and transfer to a second medium prompted either initiation of embryonic development, or as it was later thought, embryo maturation. At page 99, auxins or auxins in combination with cytokinins are said to be essential to the onset of growth and the induction of embryogenesis. At page 100, cytokinins are characterized as important in fostering somatic embryo maturation and cotyledon development. This article indicates that embryogenesis protocols have a high degree of specificity for the type of plant being regenerated. No reports of cotton regeneration are described in this article, nor is a callus initiation medium having a high cytokinin/auxin ratio followed by an embryo induction medium having a high auxin/cytokinin ratio as used in this invention suggested or disclosed.

The only prior report of cotton transformation producing whole plants known to applicant is disclosed by P. Umbeck, et al. (1987), in "Genetically Transformed Cotton (*Gossypium hirsutum L.*) Plants," Bio/Technology *5*:263-266, describing regeneration of Coker varieties 310, 312 and 5110 from hypocotyl sections transformed with a kanamycin resistance gene (*npt*II) and a chloramphenicol acetyltransferase (*cat*) gene. After incubation with *Agrobacteria*, the hypocotyl sections were placed on a medium containing equal amounts of auxin and cytokinin (0.1 µg/ml each of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-furfurylaminopurine (kinetin) as well as 5-50 µg/ml kanamycin sulfate. After tissue amplification, embryogenic tissues were transferred to a regeneration medium without phytohormones. Mature embryos (4 mm or more with cotyledon and radicle structures) were transferred to Stewart and Hsu medium (J. Stewart et al. (1977), "In-ovulo embryo culture and seedling development of cotton (*Gossypium hirsutum L.*)" Planta *137*:113-117) with indole acetic acid (IAA), 6-benzylaminopurine (BA) and gibberellic acid (GA) all at 0.1 µg/ml.

Tissue incubation was done at 30 degrees C. No regeneration of genotypes other than Coker was reported. This article characterizes the inability to regenerate plants as a major obstacle to practical application of transformation techniques. The discussion of this article does not constitute any representation that it constitutes prior art which may be properly applied against the claims hereof.

Prior reports of cotton regeneration include the following:

J. Stewart et al. (1977) *supra*, disclose the culture of zygotic embryos of *Gossypium hirsutum L.* cv. Hancock on the high-salt media BT and BTP (the latter containing phytohormones) of C.A. Beasley, et al. (1973) Amer. J. Bot. *60*:130 modified by the addition of ammonium ion. No regeneration from somatic tissue was reported.

The first report of regeneration of a domestic cotton variety was that of G. H. Davidonis, et al. (1983), "Plant Regeneration from Callus Tissue of *Gossypium hirsutum* L.," Plant Science Letters *32*:89-93, reporting the regeneration of plants from Coker 310 genotype callus derived from culture of seedling cotyledon. Callus initiation was done on LS medium (E.M. Linsmaier et al. (1965) Physiol. Plant., *18*:100) containing 2 mg/l α-naphthalene acetic acid (NAA) and 1 mg/l kinetin. After three months callus tissue was subcultured on modified LS medium containing 30 g/l glucose, 1 mg/l NAA and 0.5 mg/l kinetin. Over a three-year period a few pro-embryoids were formed. This article reports increased embryogenic potential of callus tissue after growth on media without hormones, and that embryoid growth was slower in media lacking hormones than containing NAA and kinetin. The three-year embryo induction period and low efficiency of embryo formation indicate that this article is describing an adventitious observation. This article does not provide a teaching or protocol enabling practical, usable somatic embryogenesis and regeneration of cotton and discusses only the Coker 310 genotype.

Prior difficulties in regeneration of cotton varieties other than Coker are illustrated by the disclosure of R. C. Shoemaker, et al. (1986), "Characterization of somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.)," Plant Cell Reports *3*:178-181. This article reports evaluation of seventeen cultivars on three callus initiation and three callus maintenance media. Seedling hypocotyl sections were used. The best initiation medium was a medium containing MS salts with 2 mg/l indole-3-acetic acid (IAA), and 1 mg/l kinetin. The best maintenance medium was one containing MS salts with 10 mg/l N6-(isopentenyl)-adenine (2iP) and 1 mg/l NAA. This medium produced the most vigorous and healthy calli but was not embryogenic. The maintenance medium was found not to be necessary for induction of embryogenic callus, and callus was initiated directly on MS medium with 3% glucose and 2 mg/l NAA and 1 mg/l kinetin, then switched to the

same medium using 3% sucrose instead of 3% glucose for induction. Only Coker 201 and 315 varieties could be regenerated.

H. J. Price, et al. (1979), "Somatic Embryogenesis in Suspension Cultures of *Gossypium klotzchianum* Anderss," Planta *145*:305-307, purports to comprise the first report of reproducible somatic embryogenesis in a species of the genus *Gossypium*. The species, however, is a wild-type, nonfiber-producing species rather than a domestic cotton species. The explants used were seedling hypocotyls. The use of a "pre-culture" containing a high cytokinin concentration (2iP (N^6-(2-isopentyl)-adenine) at a concentration of 10 mg/l) was disclosed as useful prior to making a suspension culture of the callus in media containing 0.1 mg/l 2,4-D and 20 g/l sucrose, but no cytokinin. This "pre-culture" was done following culture on an MS medium containing 2.0 mg/l IAA, and 1.0 mg/l kinetin. It was found that it was essential to somatic embryo formation that after suspension culture, the cells be transferred to a B5 medium containing glutamine (a medium also containing 0.5 mg/l 2,4-D was used), and that when the "pre-culture" with high 2-iP was not used prior to the suspension culture somatic embryos did not form. The authors stated that further testing would be required to determine if other cytokinins than 2-iP or lower concentrations would be effective in "pre-cultures". This article does not disclose the regeneration of domestic varieties of cotton, nor does it disclose a protocol involving a high cytokinin/auxin callus initiation medium followed by a high auxin/cytokinin embryo induction medium.

J. J. Finer et al. (1984), "Initiation of callus and somatic embryos from explants of mature cotton (*Gossypium klotzchianum* Anderss," Plant Cell Reports *3*:41-43 describes unsuccessful attempts to regenerate plants from embryos produced from stem and petiole sections of the above wild species. High 2iP media were used followed by suspension culture in a medium containing glutamine and 2,4-D in which embryos were induced. Embryo development took place in auxin-free media. Embryos were abnormal, and efficiencies were low.

R. H. Smith, et al. (1977), "Defined Conditions for the Initiation and Growth of Cotton Callus in Vitro In *Gossypium arboreum*," In Vitro *13*:329-334 describes nutrient media useful for callus proliferation and subsequent growth of subcultures. Seedling hypocotyl explants were found superior to cotyledon or leaf explants. MS media containing IAA (2 mg/l) and kinetin (1 mg/l) were found best of the combinations of auxins and cytokinins tested for callus proliferation, and media containing 2 mg/l NAA and 0.5 mg/l BA, or 1 mg/l NAA and 5-10 mg/l 2iP were found to be best for subculture. The authors report one adventitious case of plantlet regeneration (no further details available) which they did not pursue. This disclosure was not directed to somatic embryo production.

Reproducible regeneration protocols with respect to fiber-producing cotton species have thus been limited to Coker varieties of *G. hirsutum*.

T. L. Reynolds (1986), "Somatic Embryogenesis and Organogenesis from Callus Cultures of *Solanum Carolinense*," Amer. J. Bot. 73:914-918 describes culture of stem segments of a species of horse-nettle on a medium supplemented with 10 mg/l 2,4-D and 1 mg/l kinetin for callus initiation, with subculture on a medium lacking 2,4-D but containing a cytokinin for embryo production and regeneration. These protocols directly teach against the protocols used by applicant herein involving a callus initiation medium having a high cytokinin/auxin ratio and an embryo induction medium having a high auxin/cytokinin ratio.

Applicant co-authored a poster displayed at a conference at the University of California at Davis, California August 24-29, 1986 on "Tailoring Genes for Crop Improvement" entitled "Transformation and Regeneration of Cotton, *Gossypium hirsutum* L." This poster described transformation and regeneration of cotton.

In a nonenabling abstract for the Thirty-eighth Annual Meeting of the Tissue Culture Association held May 27-30, 1987, entitled "Transformation of Cotton (*Gossypium hirsutum* L.) by *Agrobacterium tumefaciens* and Regeneration of Transgenic Plants," applicant and others report transformation and regeneration of cotton. An oral presentation on the subject was made by applicant.

Zhou, G.-Y. *et al.* (1983), "Introduction of Exogenous DNA into Cotton Embryos," Meth. Enzymol. *101*:433-481 discloses a method for injection of DNA from *G. barbadense* into *G. hirsutum* ovaries. Mutations were observed in progeny, however, this method does not allow for the transformation of plants with selected foreign genes governing particular desired traits as do the methods of this invention.

Figure 4 is a diagram illustrating a regeneration scheme for cotton of this invention. The abbreviations GO through G3' $MS_{zn\text{-}g}$, $1/2G_0$ and GRM_{gn} are described in Table 14 hereof.

A process is provided for regenerating a whole plant of the genus *Gossypium*. Preferably the plant is of a fiber-producing species selected from the group consisting of *G. arboreum*, *G. herbaceum*, *G. hirsutum*, *G. barbadense*, *G. lanceolatum*, G. *tomentosum*, *G. mustelinum*, and *G. darwini*. More preferably, the species is a cultivated fiber-producing species selected from the group consisting of the first five above-named species. Of these, *G. hirsutum* is the most useful for cultivation in the United States and regeneration of this species is a preferred embodiment of this invention.

As discussed above, the Coker varieties in general, and in particular, Cokers 310, 312, 5110, 201, and 315 are regenerable by means of prior art techniques, i.e. the use of a primary medium for callus initiation containing low concentrations of auxins (.1 mg/l to 10 mg/l and equal or lower concentrations of cytokinin, and a secondary medium containing the same hormones or no hormones for embryo induction or maturation). Other varieties of *G. hirsutum* were not responsive to these techniques. Easily regenerable varieties such as Coker are termed "Class 1 varieties" herein. A "Class 1 variety" is one regenerable by means of a basic somatic embryogenesis protocol as described by Ammirato, P. V., *supra* involving a primary medium with an auxin source and a secondary medium devoid of growth regulator or having cytokinins. Class 1 varieties respond to the callus initiation, embryogenic callus induction and embryogenic callus formation steps of the protocol of this invention much sooner than other, more hard to regenerate cotton varieties which are designated as "Class 2 varieties". Class 2 varieties are not regenerable by prior art methods and respond more slowly to the protocol of this invention than Class 1 varieties, all as described below.

Tissue from the plant to be regenerated is first placed on a callus initiation medium. Tissue explants useful in practicing this invention include hypocotyl, cotyledon and leaf sections, preferably taken from precociously germinated seedlings. Hypocotyl segments were especially useful in obtaining regeneration of Class 2 varieties; however, cotyledon sections are also useful. Immature embryos, or portions thereof may also be used. Immature embryos are fully developed but not yet hardened.

The preferred embodiment of this invention involves the use of a callus initiation medium having a high cytokinin to auxin ratio to proliferate callus. A number of cytokinins are well known to the art, and more fully described below. The most preferred cytokinin of this invention is 2iP. Auxins are well known to the skilled worker and described in the prior art as well. The preferred auxin for use in this invention is NAA. A high cytokinin auxin ratio is defined herein to be greater than about 10:1; preferably the ratio is at least about 30:1 to 50:1 and can be as high as 100:1. The cytokinin concentration may be as high as about 10 mg/l, but not less than about 1 mg/l, and the auxin concentration should not be more than about 1 mg/l and may be as low as about 0.01 mg/l.

The callus initiation culture is continued until the callus has proliferated to about 5-10 times its original size, and until the calli are sufficiently mature that the auxins will not be toxic to the cells, generally about two to three weeks for the Class 1 varieties and about four to five weeks for the Class 2 varieties.

After the callus initiation step, the callus is transferred to an embryogenic callus induction medium having high auxin to cytokinin ratio. A "high" ratio of auxin to cytokinin for this purpose is defined as being at least about 1:1. Preferably this ratio is at least about 3:1 and more preferably about 50:1 to about 100:1. The auxin concentration should not be more than about 5 mg/l or less than about 1 mg/l, and the cytokinin concentration should not be more than about 1 mg/l and can be as low as 0.0 mg/l. The tissues are maintained on this medium until embryogenic callus is induced, characterized by production of proembryoids having globular and heart-shaped structures --generally about two to three weeks for Class 1 varieties and about five to six months for Class 2 varieties.

The calli are then transferred to suitable media for embryogenic callus formation, embryo maturation, embryo germination, and plant regeneration.

In a preferred embodiment of this invention utilizing the above preferred protocol, the regenerated plant is a Class 2 cultivar.

Also in a preferred embodiment of this invention, following the embryogenic callus induction stage, the calli are transferred to an embryogenic callus formation medium without phytohormones for maximum embryogenic callus production.

Culturing on the hormone-free medium is preferably continued until embryo maturation occurs, i.e. somatic embryos have a pair of cotyledons, green color and are about three to about 12 mm in size. The calli can be maintained on this medium for as long as desired. Preferably, however, after about three to four weeks for Class 1 varieties and about six to seven weeks for Class 2 varieties, the calli are transferred to a new medium for another two to three weeks. This new medium is preferably hormone-free medium, but optionally, may be medium containing small amounts of auxin, preferably NAA at about 0.1 mg/l, a cytokinin, preferably zeatin at about 1 mg/l, and reduced carbohydrate concentration, preferably about 1.5% glucose. The preferred hormonal medium for this purpose is $MS_{zn\text{-}g}$ as shown in Table 14.

Embryo germination media for use following the embryo maturation step described above are known to the art. A preferred embryo germination medium is a low ionic strength medium such as Stewart and Hsu medium without the added ammonium. Preferably this medium is supplemented with a gibberellin and an auxin, preferably about 0.1 mg/l gibberellic acid and about 0.01 mg/l NAA, and preferably glucose (0.5%) is used instead of sucrose.

As shown in Figure 4, in a preferred embodiment hereof, after about two to three weeks on the preferred embryo germination and plantlet development medium, GRM_gn, the plantlet is transferred to a plant development medium such as a phytohormone-free medium containing reduced

salts and carbohydrate (preferably about 1/2 MS salts and about 1.5% glucose) or culture may be renewed on a germination medium, preferably GRM_{gn}.

Growth to a whole fertile plant is continued under greenhouse conditions.

The regeneration protocol provided herein is particularly valuable for use in genetic engineering to produce whole, fertile transformed cotton plants. Transformation (incorporation of foreign DNA into the plant genome) may be accomplished by any means known to the art, preferably by infection with *Agrobacterium tumefaciens* containing the desired foreign DNA. The transformed tissues are cultured as above for regeneration into whole plants. "Foreign" refers to any DNA or genes which do not occur naturally at their new location in the host plant's genome. Foreign genes may be genes with their own promoters or chimeric genes derived from *Gossypium* or other organisms. Preferably, the foreign DNA or genes confer an identifiable phenotype on the regenerated host plant and/ or its progeny by which the plant is distinguishable from naturally occurring plants. Such phenotypes conferred by foreign DNA include performance on laboratory tests such as Southern, northern and western blot procedures. Also preferably the transformation method allows for the insertion of selected genes or DNA into the cotton genome. A "selected gene" is a gene governing a particular trait which it is desired to confer on the recipient plant (as distinguished from unselected genes which may be transferred by methods such as injection of DNA extracted from other organisms and containing an unknown type and quantity of genes). Other types of "selected" foreign DNA might be particular isolated promoters or enhancers transferred to the recipient genome to perform their known functions.

By means of this invention, whole transformed cotton plants, preferably Class 2 varieties, are obtained which can express the foreign DNA or genes contained therein, e.g. foreign promoters and enhancers can be expressed to operate to turn on or enhance the activities of other genes, and foreign genes can be expressed to produce RNA and protein.

By means of this invention a regenerated plant preferably of a Class 2 *Gossypium* genotype transformed to contain foreign DNA and having a phenotype conferred by said foreign DNA by which said plant can be distinguished from a natural occurring plant is produced. Progeny of these plants may also be produced, as well as seeds of said plants and progeny plants. Any plant produced by the methods of this invention which is not phenotypically distinguishable from a naturally-occurring plant, is nevertheless considered to be within the scope of equivalents of plants claimed herein which are phenotypically distinguishable.

This invention has provided an important improvement in methods for regenerating cotton plants, which methods involve culturing somatic tissue of said plants on suitable media to cause callus formation and whole plant regeneration, the improvement of this invention comprising using somatic tissue of a Class 2 genotype of a *Gossypium* species.

COTTON REGENERATION EXAMPLE

Tissue from a plant of genus *Gossypium*, preferably a fiber-producing species thereof, and more preferably, a species of *G. hirsutum*, is regenerated to produce a whole plant. In the preferred embodiment hereof, the genotype used is a Class 2, difficult-to-regenerate genotype, preferably a genotype of *G. hirsutum*, and more preferably a GSA genotype.

The tissue is preferably obtained from seedlings about seven to about 10 days old, and preferably the seedlings are grown from immature seeds taken from cotton bolls which are about three to about five centimeters in diameter, about 40 to about 60 days after pollination. Incubation procedures for growing up seedlings are known to the art, for example as described in Firoozabady, E., *et al.* (1986), IC "Isolation, Culture, and Cell Division in Cotyledon Protoplasts of Cotton (*Gossypium hirsutum* and *G. barbadense*)," Plant Cell Rep. *5*:127-131. The medium used for production of seedlings is preferably a G_0 medium not containing hormones as described in the Cotton Regeneration and Transformation Examples hereof.

The explants used for tissue culture are preferably cotyledon pieces, preferably approximately 0.6 cm^2 in surface area, or hypocotyl sections, preferably about 5-8 mm in length, or leaf pieces approximately 0.6 cm^2 in surface area, taken from seedlings. These explants are taken when the seedlings are large enough to provide tissue of sufficient size, and preferably the seedlings are approximately three weeks old when the explants are taken.

Seedlings may be grown from immature seeds or germinated by culturing dry seeds. Tissue from immature (fully developed but not hardened) embryos may also be used.

The initial culture medium (callus initiation medium)is a medium containing a high cytokinin/auxin ratio. The cytokinin concentration must not be so high that it is toxic to plant cells, but must be sufficiently high that it stimulates growth of plant cells, and the auxin concentration must not be so high that it is toxic, but must be sufficiently high that it induces cell proliferation. Preferably the cytokinin concentration is between about 1.0 and about 10.0 mg/l, and more preferably between about 3 and about 5 mg/l. The auxin concentration is preferably between about 1 and about 0.01 mg/l, and more preferably between about 0.1 and about 0.2 mg/l. The cytokinin to auxin ratio is

preferably between about 10:1 and about 100:1, and more preferably between about 30:1 and about 50:1. The most preferred callus initiation medium is the G_2 medium described in Table 14 containing 5 mg/l 2iP and 0.1 mg/l NAA along with MS salts (Gibco, Grand Island, New York), and glucose at 3.0% (w/v), along with 100 mg/l myo-inositol, 0.4 mg/l thiamine HC1, 0.2% Gel-rite (Kelco, San Diego, California), at pH 5.9. As will be understood by those skilled in the art, other basal media and carbohydrate sources may be substituted for those specifically described herein. Other basal media known to the art as useful for regeneration are SL, V5 and L2. Preferably the auxin is from the NAA family, defined herein to include IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), and NAA (alpha-naphthaleneacetic acid). Other auxins known to the art are 2,4-D and related auxins of the 2,4-D family, defined herein to include 2,4-D (2,4-dichlorophenoxyacetic acid), Picloram (4,amino-3,5-6-trichloropicolinic acid), pCPA (parachlorophenoxyacetic acid), 2,4,5-T(2,4,5-trichlorophenoxyacetic acid), and Dicamba (2-methoxy,3-6-dichloro-o-anisic acid). Many cytokinins are known to the art. Examples of useful cytokinins are ADE (adenine sulfate), kinetin (6-furfurylaminopurine), BA (6-benzylaminopurine), and zeatin. Concentrations of 2,4-D higher than about 0.1 mg/l, however, have been found to be toxic to cotton cells, and 2,4-D is thus not a preferred auxin.

This initial high cytokinin culture is essential to achieving the efficiency of embryo induction in later culture stages required for a practical cotton regeneration method.

The callus produced on the callus initiation medium should be midfriable, rather than extremely hard (nonfriable) or extremely friable, as midfriable callus gives the best production of embryogenic callus in subsequent culture steps.

For callus initiation, a high temperature, preferably around 30°C is preferred, but is not critical. Light intensity during this phase of regeneration does not appear to be critical, and may vary from complete darkness up to high intensities, such as 90 $\mu E/m^2/s$.

After about two to about three weeks of callus initiation for Class 1 varieties (e.g. Cokers), or about seven to about nine weeks for Class 2 varieties (e.g. GSA'S), the calli are transferred to an embryogenic callus induction medium. This step is essential for regeneration of Class 2 varieties but may be omitted for Class 1 varieties. This medium contains a high auxin/cytokinin ratio. The auxin concentration should be high enough so that it stimulates the process of embryogenesis, but not so high that it is toxic to plant cells; preferably the auxin concentration is between about 1 and about 5 mg/l, and more preferably between about 3 and about 5 mg/l. The cytokinin concentration should be high enough so that it induces embryogenesis, but not so high that it prevents embryogenesis.

Preferably the cytokinin concentration is between about 0 and about 1 mg/l and more preferably between about 0.05 and about 0.1 mg/l. In the preferred embodiment described in the Example, media containing NAA at 5 mg/l and 2iP at either 1 or 0.1 mg/l, and also containing the additional components described above in connection with the G_2 medium are used. These two media are respectively G_1 and G_3. Again, as will be understood by those skilled in the art, equivalent components may be used, but it is necessary to maintain a high auxin/cytokinin ratio in this medium. Glucose is the preferred carbohydrate for use in the embryogenic callus induction medium, as well as for all the culture media used in this invention, as sucrose promotes production of phenolics and calli cultured on this medium have been observed to turn brown, and eventually to die. G_3, described in Table 14, is the preferred embryogenic callus induction medium.

Embryogenic callus formation may be observed on the embryogenic callus induction medium when the culture is kept on the medium long enough to exhaust the hormones. Preferably, however, the callus is transferred to hormone-free medium for embryogenic callus formation.

In the preferred embodiment after about three to four weeks on the embryogenic induction medium for the Class 1 genotypes, or about four to five months for the Class 2 genotypes, the callus is transferred to a phytohormone-free medium for embryogenic callus formation. Stages of somatic embryo development are well defined in the art, e.g. by Shoemaker, R. C., *et al.* (1986), *supra*, and Finer, J. J., *et al.* (1984) , *supra*. Immature somatic embryos lack well defined organs, whereas normal, mature somatic embryos are those with a pair of cotyledons and normal morphology (i.e., green color and about three to about 12 mm in size). The hormone-free medium used for embryogenic callus formation is preferably the medium used for callus initiation or for embryogenic callus induction without the phytohormones, preferably G_1, G_2 or G_3 medium without the hormones. As will be apparent to those skilled in the art, however, other hormone-free nutrient medium may be used.

Embryogenic calli are recovered at a frequency of about 10% to about 85% depending upon the genotype used, the Class 1 genotypes producing calli faster and at higher frequencies. Embryogenic calli are characterized as midfriable, creamy, granular tissues. They are generally observed first as sections of calli or sometimes whole brown, midfriable calli become embryogenic. This embryogenic callus contains proembryoids (globular and heart-shaped structures).

For embryogenic callus formation, high temperature (e.g. around 30°C), and low light, for example, about 9 $\mu Em^2/s$, are preferred.

The embryogenic callus after about two to three weeks on the embryogenic callus formation medium for a Class 1 variety or about four to six weeks for the Class 2 varieties, is subcultured on the same medium, or on a medium containing a cytokinin, preferably zeatin, an auxin, preferably NAA, and reduced carbohydrate. Preferably the auxin is present at between about 0 and about 0.2 mg/l, more preferably between about 0.01 and about 0.1 mg/l, and preferably the cytokinin is present at between about 0.5 and about 3.0 mg/l, and more preferably at a concentration between about 0.5 and about 1 mg/l. The medium $MS_{zn\text{-}g}$ as described in the Examples hereof is the most preferred of such supplemented media. This medium contains 0.1 mg/l NAA and 1 mg/l zeatin together with MS salts, 1.5% (w/v) glucose, and the other nonhormone components of the G1, G2 and G3 media. For this final embryo maturation step, however, the nonsupplemented medium G_0 is most preferred. The use of 2,4-D or pCPA as the auxin in the supplemented medium, or reduction of the MS salts to one-half strength in either medium reduced the capacity of the medium for embryo production and maturation.

Mature embryos vary in size from about 2 to about 12 mm, have between about one and about four cotyledons, have variable hypocotyl lengths and vary in color from pale yellow to light green to dark green and occasionally albino most likely due to tissue culture art (but this albinism is not a heritable character).

After about two to three weeks on the embryo maturation medium for both Class 1 and Class 2 genotypes, mature embryos are transferred to an embryo germination and plant development medium. Germination media are known to the art, for example as described by Stewart, J. M. *et al.* (1977), *supra*. The germination medium may be modified by the addition of gibberellic acid (GA_3) at a concentration of between about 0 and about 1.0 mg/l, and preferably at a concentration of between about 0.05 and about 0.2 mg/l. The germination medium may also be modified to contain an auxin, preferably NAA, at a concentration of between about 0.0 and about 0.1 mg/l, and preferably not more than about 0.05 mg/l. Also preferably the medium uses glucose rather than sucrose, preferably at a concentration of between about 0.2 and about 1.5% (w/v), and more preferably at a concentration at between about 0.5 and about 1.0%. The most preferred medium is the medium described herein as GRMgn, containing about 0.1 mg/l gibberellic acid, about 0.01 mg/l NAA and about 0.5% glucose. The preferred medium is a low ionic strength medium. Ionic strength equal to the use of MS salts at a concentration of about 1 X MS salts caused burning and senescence of the tissues and caused problems in balancing root and shoot formation. The preferred medium will allow germination of at least about half of the mature somatic embryos. Iron at a concentration of between about 10 μm and

about 25 μm is an important component of the germination medium. Embryo germination or "conversion" is defined as the development of the apical area of the somatic embryo resulting in shoot production (true leaves).

Embryos developed well on the GRM_gn medium, as they do not tolerate higher salt concentrations well. If desired, a compound such as asparagine or ancymidol may be added to the GRM_{gn} medium. Plantlets grown on media containing these compounds have a dark green color and good root systems. These compounds may be added in concentrations which will be apparent to the skilled worker, preferably about 5 ppm ancymidol or about 100 mg/l asparagine.

High light intensity, (e.g. about 90 $\mu E/m^2/s$) are helpful for germination and plantlet development. During the embryo germination and plantlet development phase, it is also preferable to initially incubate mature embryos at high temperatures, e.g. about 30°C, for a few days to produce rapid germination, then lower the temperature to normal, about 25°C, for plantlet development.

After about one to two weeks on the embryonic germination and plantlet development medium, the plantlets, defined herein as germinated embryos having roots as well as shoots, may be transferred to media known to the art for further plant development. Preferably the plantlets and germinated embryos are transferred to Magenta cubes, such as GA7 Magenta cubes (Magenta Corporation, Chicago, Illinois), containing a phytohormone-free medium with reduced salts and carbohydrates, preferably about one-half MS salts and about 1.5% glucose. When plants are partially developed, e.g., about 8 to about 10 cm tall, having about 4 to about 6 leaves, they may be transferred to soil for maturation. Preferably the plants in soil are grown under initial high relative humidity for a gradual hardening off, and then under normal greenhouse conditions.

The foregoing procedure allows regeneration of full plants within about 14 to about 16 weeks for Class 1 varieties and about 8 to about 10 months for Class 2 varieties.

A well-defined, reproducible, and highly efficient plant regeneration scheme such as that defined above, is a prerequisite for transformation of cotton. Transformation may be performed by means known to the art for introduction of foreign DNA into plant cells and tissues. Once transformation of cells and/or tissues has been done, the transformed cells and tissues can be regenerated according to the above-described methods.

A preferred method of transformation involves the use of *Agrobacteria* to introduce foreign DNA into plant cells via infection. Means for inserting foreign DNA for transfer to plant tissues into *Agrobacteria* are well known to the art, and many vectors carrying such genes are known and readily available to the skilled worker. In a preferred embodiment, a coding region for the insecticidal

crystal protein (Bt) from *Bacillus thuringiensis* is used. Vectors containing Bt are described, for example, in U.S. Patent Application Number 848,733, incorporated herein by reference, and in Patent Application Number 617,321, also incorporated herein by reference.

The vector preferably contains a "marker" gene such as the neomycinphosphotransferase II (*npt* II) gene conferring kanamycin resistance. Other suitable markers are known to the art.

The *Agrobacteria* containing the vector to be used to insert foreign DNA into the plant tissue is cultured by means known to the art, preferably by growing on appropriate media such as agar media containing selection agents corresponding to the marker genes present on the vector, e.g., streptomycin and chloramphenicol. Other media, such as YEP medium, may also be used.

The *Agrobacterium* colonies are scraped off the selection medium and suspended in an appropriate liquid medium, such as YEP broth or minimal medium. Preferably, however, the bacteria are suspended in a liquid medium for cotton callus culture, preferably a callus initiation medium such as G_2 medium as described above, preferably used at a concentration of about 2-4 x 10^8/ml. The tissues are submerged in the bacterial suspension to assure adequate contact of the tissue to be transformed with the *Agrobacteria*. Concentrations are preferably less than 10^9 or 10^{10}, as such high concentrations tend to kill the tissue.

The tissue to be transformed may be any regenerable cotton tissue. Preferably cotyledon, hypocotyl and leaf sections from seedlings developed from immature embryos or germinated seedlings as described above are used. The tissue pieces may be of any manipulable size, and preferably are about 1/2 cm^2.

The tissue pieces are contacted with the *Agrobacterium* suspension, preferably by dipping in the liquid culture medium and shaking to ensure contact of all edges with the culture. To minimize *Agrobacterium* on the tissue, blotting dry, preferably with filter paper, is recommended. This reduces bacterial overgrowth on the plant tissues.

The tissue are co-cultivated with the *Agrobacterium*, preferably at about 25°C, and preferably under low light (10 $\mu E/m^2/s$), on a suitable medium, preferably a callus initiation medium such as G2 for long enough to ensure infection, preferably about two to three days. Preferably the tissue is plated on filter paper placed on the medium for co-cultivation to reduce bacterial overgrowth.

The infected tissues after co-cultivation are then placed on a medium to kill the *Agrobacteria*, for example by containing antibiotics known to the art. Examples of such antibiotics include carbenicillin, cloxacillin, and cefoxitin and preferably about 500 mg/l carbenicillin is used. The medium should also contain a selection agent to select for transformed tissue, corresponding to the

marker gene present in the vector. The selection agent should be present at a concentration high enough that untransformed cells do not grow, but not so high as to kill the transformed tissue. The selection agent used in the Examples is kanamycin sulfate, at a concentration of between about 15 and about 40 mg/l, preferably about 25 mg/l. Concentrations above about 50 mg/l, especially in the 100 to 150 mg/l range nonselectively kill the cells. Other selection agents known to the art, such as G418, hygromycin, bleomycin, and methotrexate are also useful. It is important that all cells have contact with the medium so as to ensure selection.

After a sufficient period of time to ensure selection and initiation of transformed microcalli, generally about one to about three weeks, the tissue is regenerated as described above. Preferably when microcallus grows to about 3 to about 4 mm, it is excised from the original explant and transferred to fresh medium. The time required for regeneration of transgenic plants, that is, the plants that contain the foreign DNA, is longer than for nontransferred plants due to inoculation of tissues with *Agrobacteria*, the selection pressures and effects of antibiotics.

All regenerable cotton varieties as described above may be transformed by the above methods, and by other methods known to the art, for example as described in S. H. Mantell, *et al.* (1985), Principles of Biotechnology, particularly chapter 4 thereof and the references referred to therein.

Dry seeds of *G. hirsutum* cultivars (Coker 201, 310, 315 and 4360; GSA 71, 75 and 78; GSC 25 (GSA and GSC are cultivars developed by GroAgri Seed Company (a subsidiary of Agrigenetics Corporation), Lubbock, Texas); G8160 (a breeding line from US Cotton Research Station, Shafter, California); GSA-Acala hybrids No. 21 and No. 22 (No. 21 is Acala SJ-C1 X GSC 20 and No. 22 is Acala SJ-Cl X GSA 74-7, 127 hybrid); and Acala SJ-2) were surface sterilized as described (Firoozabady, E. and DeBoer, D.L. (1986) Plant Cell Reports *5*:127-131, except that seeds were exposed to bleach for only 8-10 minutes, and germinated on G_O medium. Composition of media used in cotton regeneration is presented in Table 14 unless published elsewhere or modifications are mentioned in the text. Immature seeds were obtained from cotton bolls (3-5 cm diam., 40-60 days after pollination) of Coker 201 and 315 and GSA cultivars. Bolls were surface sterilized 20 minutes in 33% commercial bleach and rinsed twice in sterile distilled water. Immature seeds were manually delinted, seed coats were
removed, and the embryos (7-10 mm) were germinated on G_O medium and incubated as described in Firoozabady and DeBoer, (1986), *supra*.

Cotyledon pieces (approximately 0.6 cm^2 surface area) and hypocotyl sections (5-8 mm length) of 7- to 10-day-old seedlings and leaf pieces (approximately 0.6 cm^2 surface area) of 21-day-old plants were placed on callus initiation medium, G_2. Only hypocotyl sections were cultured from precociously germinated seedlings. After two to three weeks for Cokers or seven to nine weeks for GSA's, calli were transferred onto embryogenic callus induction media (G_1' G_3). Embryogenic calli were transferred to G_0 or MS_{zm-g} medium to produce mature somatic embryos. These were germinated on the medium suggested by Stewart and Hsu (1977), *supra*, modified by addition of 0.1 mg/l GA_3 (filter-sterilized), 0.01 mg/l NAA and 0.5% glucose instead of sucrose (GRM_gn). After one to two weeks, the germinated embryos were transferred to $1/2G_0$ or GRM_gn in GA7 Magenta cubes (Magenta Corp., Chicago, IL) to develop further. The plants (10-12 cm tall, 5-10 leaves) were transferred to soil under high relative humidity, gradually hardened off, and transferred to normal greenhouse conditions.

To study the effects of temperature and light intensity on different stages of tissue culture, tissues were incubated at 25±1°C or 30±1°C under high light intensity (90 $\mu E/m^2/s$) or low light intensity (9 $\mu E/m^2/s$) provided with cool white fluorescent lamps (GTE, Salem, MA) . Also, the effects on embryo quality and germination of different media containing additives such as ancymidol (Elanco Products, Co., Indianapolis, IN), asparagine, or different glucose concentrations were examined.

Immature somatic embryos were characterized by lack of well-defined organs. Normal somatic embryos are those with a pair of cotyledons and normal morphology (i.e., green color and 3-12 mm in size).

Results were as follows:

Callus initiation and proliferation. A range of media were tested for callus initiation from several cultivars. Medium G_2 (5 mg/l 2iP and 0.1 mg/l NAA, Table 14) was best for callus initiation and growth in many cultivars tested. Medium G2 was superior to G1, G3, EF18 (2 mg/l NAA and 1 mg/l kinetin, Shoemaker, R. C. *et al.* (1986) *supra.*), and the medium containing 2 mg/l IAA and 1 mg/l kinetin used by Smith R. H. *et al.* (1977) *supra.* for *G. arboreum* callus initiation. Including MS vitamins and/or 5% (v/v) coconut milk in some media such as EF18 were helpful in overall callus growth, but still these were inferior to G_2 medium.

SMpi medium (7.5 mg/l 2iP and 0.1 mg/l pCPA, Firoozabady, E. (1986), *supra* and SMgpi (SMpi with glucose instead of sucrose) were good for callus proliferation and maintenance, but still inferior to G2. Relatively hard, creamy granular calli were produced on media SMpi and SMgpi.

Rapid callus initiation on G_2 medium and proliferation on SMpi or SMgpi media indicating high level of 2iP or high 2iP/auxin ratio is important in these processes.

A range of gross morphology of the initiated calli was observed and varied from hard (nonfriable) to extremely friable, midfriable being the desired morphology. The degree of friability was highly dependent on the hormones used. For example, less friable tissues were obtained with higher levels of 2iP and higher 2iP/auxin ratios. Friability generally increased with higher NAA concentrations. Inclusion of 2,4-D in the media resulted in production of hard, compact calli. When no hormone was included in the subculture medium, midfriable tissues were obtained.

Embryogenic callus induction. Embryogenic calli were characterized as midfriable creamy granular tissues. Embryogenic calli were observed first as sections of calli or sometimes whole brown midfriable calli became embryogenic. These contained proembryoids (globular and heart-shaped structures). Transferring calli from EF18 to EFs18 (EF18 containing sucrose instead of glucose) was predicted by Shoemaker, R. C. *et al.* (1986) *supra.* to induce embryogenic callus formation. With Coker 201 and 315, this was possible at a very low frequency (2-3%, Table 15), but other cultivars did not respond to this exchange of carbohydrates. Calli on EFs18 (and other sucrose containing-media) generally produced a lot of phenolics, turned brown arid eventually died. With the subculture medium used by Davidonis, G.H., *et al.* (1983) *supra.*, only Coker 201 and 310 produced embryogenic calli (data not shown). In this work, media G1 and G3 containing 5 mg/l NAA produced massive amounts of embryogenic calli over several cultivars tested (Table 15), indicating that NAA is important for embryogenic callus induction. The fact that G3 was better than G1 also indicates that a high NAA/2iP ratio is beneficial for embryogenic callus induction in cotton.

Use of germinated immature seeds as the source of explants resulted in a higher rate of embryogenic callus formation in GSA lines but not in Coker 201 and 315.

Maturation and germination of somatic embryos and plant regeneration. After three to four weeks (Cokers) or approximately four months (GSA'S) on embryogenic callus induction medium (G_1 or G_3), embryogenic calli containing globular through torpedo stage somatic embryos were transferred to hormone-free (G_0) medium or sometimes to zeatin/NAA (MS_{zn-g}) containing medium. This subculture resulted in maximum embryogenic callus production (Table 15) and within two to three weeks, numerous tulip-shaped and mature embryos developed. Medium G_0 was best for somatic embryo maturation with several cultivars (Coker 201, 310, and 4360); GSA 78; and hybrid No. 21) tested. MS_{zn-g} was the second best in this regard. Replacing NAA with 2,4-D or pCPA in MS_{zn-g} media, greatly reduced their capacity for embryo production and maturation.

Embryos were very different in size (2-12 mm range) and morphology. They had different numbers of cotyledons (1-4), hypocotyl length, and colors (dark green, light green, and pale yellow).

Somatic embryos were transferred onto a lower ionic strength medium (GRM_{gn}) to germinate. GRM_{gn} was the best medium for embryo germination and plantlet development (Tables 16 and 17). Eliminating GA_3 and NAA reduced germination frequencies of the somatic embryos (GRM, Table 16). Generally, approximately 50% mature somatic embryos (normal or abnormal) germinated on GRM_{gn} (Tables 16 and 17). Larger embryos (8-12 mm, mature)germinated more frequently (approximately 90%) than small embryos (2-4 mm, immature). Usually G_1/G_3 - G_0 - GRM_{gn} resulted in higher frequencies of embryos germination and plantlet development than G_1 - GRM_{gn}, G_3 - GRM_{gn}, or any other transfer regimes (Table 17). This was probably due to higher frequencies of normal embryos developed on G_0 medium. Most germinated embryos produced both roots and leaves (plantlets). At the time of this writing in Coker 201, 255; Coker 310, 2; Coker 315, 6; Coker 4360, 7; No. 21, 4; No. 22, 3; GSA 71, 12; GSA 75, 20; GSA 78, 26 and GSC 30 plants have been regenerated and successfully transferred to soil and green house conditions.

Most of the regenerated plants have normal morphology, are fertile and have set seed.

Effects of light and temperature on different phases of cotton tissue culture. Different phases of cotton tissue culture were affected both by light and temperature, although these factors had less influence than medium composition. For callus initiation, a high temperature (30°C) was preferred and light intensity did not play an important role. Calli have been initiated and maintained at the same rate in complete darkness and in different light intensities (9-90 $\mu E/m^2/s$). For embryogenic callus formation and proliferation, high temperature and low light (9 $\mu E/m^2/s$) were preferred in the varieties tested: Coker 201 and 315 and GSA 78. After somatic embryos were formed, high light intensity (90 $\mu E/m^2/s$) proved to be very helpful for germination and plantlet development. It was also very helpful to incubate mature embryos at 30°C for a few days to rapidly germinate, then at 25°C for plantlet development. At high temperatures, the embryos and plantlets often grew slowly and callused.

Other observations on cotton regeneration. Embryogenic calli proliferated on G_6 medium (same as G_0 but with 6% glucose and 100 mg/l asparagine) with little mature embryo production, indicating the effects of high glucose and asparagine on embryogenic callus proliferation. Somatic embryos and plantlets in Coker 201 and 4360 have been induced to form callus (on G_2 medium) or embryogenic callus (on G_6 medium) and to undergo a second cycle of plant regeneration (Fig. 4) .

Inclusion of 5 rpm ancymidol or 100 mg/l asparagine in GRM medium resulted in healthier plantlets; the plantlets had dark green leaves and a better root system.

Some somatic embryos and plantlets developed abnormally. For example, occasionally, embryos were formed on previously matured embryos, some plantlets produced callus on top or on the stem, and some produced many slim shoots. However, these abnormal tissues could be induced to form callus and subsequently regenerate normally.

Some calli from GSA 78 and GSA-Acala hybrid No 21 have spontaneously regenerated shoots via organogenesis. However, to date we have only been able to regenerate plants from these shoots at frequencies of approximately 25% because of difficulties in inducing root formation.

Summary of results. A highly efficient and general method for plant regeneration in cotton, *G. hirsutum* has been developed. The method is very rapid for Class 1 cultivars (Cokers --14-18 weeks) and relatively slow for Class 2 cultivars (GSA's -- 8-10 months). For Class 2 cultivars developmental stages progressed more slowly than for Class 1 cultivars, and with lower efficiency of regeneration. Use of precociously germinated seedlings as the source of explants increased frequencies of embryogenic calli formation with Class 2 but not with Class 1 cultivars. Embryogenic cultures are very stable, and upon monthly subculture on G_0 medium, numerous somatic embryos are produced; at this writing plants are still being regenerated from 15-month-old Coker 201 and GSA 78 embryogenic callus lines.

In comparing different media formulations, it is clear that a high 2iP/auxin ratio is best for callus initiation and proliferation but must be exchanged for a high NAA/cytokinin ratio before embryogenic calli will form. Interestingly, in *G. hirsutum* the ability to form embryogenic calli appears to be influenced by NAA (this study, Davidonis, G. H., *et al.*, *supra* Shoemaker, R. C. *et al.*, *supra*). We have found that continuous subculture on high levels of 2iP suppresses morphogenesis. However, upon two subcultures each on induction medium (G_1 or G_3 and embryo formation medium (G_0) embryos are formed.

For maximum somatic embryo formation, it was essential to subculture calli hormone-free medium. The length of time on hormone-containing medium (i.e., G_1 or G_3), however, had an effect on embryo formation.

In the present study, since over 100 embryos were formed per dish, an ample number was readily obtained to test the effects of various cultural conditions on embryo germination and plant development. Germination of somatic embryos was highly dependent on frequency of normal embryos and ionic strength of the medium. Abnormal embryos rarely germinated under any

condition and had a long lag period. Small embryos also germinated infrequently and slowly. Normal and large embryos (greater than 5 mm), however, germinated well on appropriate media. Embryos placed on BT medium (Beasley, C. A., *et al.*, *supra.*) either developed callus or their growth stopped and all tissues became necrotic. Embryos were unable to tolerate high- salt (Table 16, EG) or high-sugar (Table 16, EG, EG_1) media, and *myo*-inositol was partially inhibitory to root development (Table 16, EG1, EG3). Often root or shoot development was a problem, and callusing was very common. The embryos performed best on modifications of a medium recommended by Stewart, J. N., *et al.*, *supra.*, GRM_{gn}. Addition of compounds such as asparagine and ancymidol to GRM_{gn} proved helpful in embryo development. Balanced root and shoot growth was obtained using the medium GRM_{gn}. Embryo growth was slower in the medium lacking hormones (GRM). Embryo germination frequencies were higher on GRM_{gn} medium in cases where embryos matured on hormone-free medium, G_0, due to production of higher frequencies of normal embryos on G_0 medium (Table 17). On hormone-containing media, G_1 and G_3, proembryoids grew slowly and many callused and proliferated instead of undergoing maturation.

COTTON TRANSFORMATION EXAMPLE

The neomycinphosphotransferase II (*npt* II) gene confers kanamycin resistance in eukaryotes when inserted behind eukaryotic promoters. The *npt* II coding sequence from bacterial transposon Tn5 was inserted behind the cauliflower mosaic virus (CaMV) 19S promoter and was terminated by addition of T-DNA 0RF26. This chimeric gene, T-DNA OCS gene, *npt* II gene, and A and B borders of T-DNA was inserted into the broad host range replicon pTJS75 to create pH575. The construction of this vector is more fully described in U.S. Patent Applications No. 788,984 filed October 21, 1985, and incorporated herein by reference.

Agrobacterium tumefaciens strain LBA4404 carrying pH575, and LBA4404 without the vector were cultured on Luria broth agar medium containing 250 µg/ml streptomycin and 25 µg/ml kanamycin (both from Sigma) for selection. Strain LBA4404 is described in Hoekema, *et al.* (1983) Nature *303*:179. This is a widely available strain also freely available from the authors. Bacteria were scraped off the agar medium, suspended in a liquid medium for cotton callus culture (G_2) to a concentration of 2-4 X 10^8 cells/ml and were used for inoculation of cotyledon segments.

To transform cotton tissues, cotyledon pieces (approximately 0.5 cm^2 surface area) from sterile 12- to 14-day- old Coker 201 seedlings were dipped in *A. tumefaciens* liquid cultures in Petri dishes and gently shaken for a few seconds to ensure contact of all cotyledon edges with the bacterial

cultures. The cotyledon pieces were then blotted dry and plated on Whatman #1 filter paper on a callus initiation medium (G_2)containing MS salts (Gibco), 100 mg/l *myo*-inositol, 0.4 mg/l thiamine HCL, 5 mg/l 2iP, 0.1 mg/l NAA (all from Sigma), 3% (w/v) glucose, 0.2% Gel-rite (Kelco), pH5.9. Filter paper was not necessary for transformation, but greatly reduced bacterial overgrowth on plant tissues. After three days cultivation at low temperature (25°C) and low light, cotyledon pieces were transferred to Petri plates containing the same medium, without the filter paper, and containing 500 mg/l carbenicillin and 25 mg/l kanamycin sulphate (both from U.S. Biochemicals).

After 7-10 days of incubation, cotyledon pieces initiated transformed kanamycin-resistant microcalli (0.5 mm) at wound sites, while no callus from control untreated tissues or from tissues treated with LBA4404 grew on kanamycin. Two to three weeks later, 2-4 mm calli were excised from original explants and transferred to fresh medium. All the calli were kanamycin-resistant and 80-100% of the kanamycin-resistant calli were positive when tested for octopine (Table 18). Two to three weeks later, the calli were placed and maintained on embryogenic medium G_O (same as G_2 with no hormones) under selection. Mature somatic embryos were transferred to lower ionic strength medium, GRM_{gn} described above. Plants were shown to be transformed by their resistance to kanamycin in leaf callus assay, production of octopine, enzyme-linked immunoassay (ELISA) for NPT II (Table 19) and by DNA, and western blot hybridization analyses. Plants were transferred to soil for further analysis. The whole process from infection until transgenic plants were transferred to soil took about six months. Similar results were obtained in replicate experiments.

Transgenic cotton plants are readily obtained with the transformation-regeneration system described here. The system is very efficient and has been modified to be applicable for plant regeneration of a number of commercial cultivars of cotton, although the efficiency and time period for regeneration of other cultivars are not as short as for Coker 201. The use of a selectable kanamycin-resistance marker was necessary. Kanamycin-resistant microcalli developed at the wound sites. In the absence of selection, barely detectable octopine-positive calli were obtained in frequencies of 10-20% indicating transformation frequencies were reasonably high among the cell populations of some calli.

Generally, 150-200 calli per 100 cotyledon sections were produced on kanamycin; 100% of these survived during subculture and remained to be resistant to kanamycin; about 80-100% of these were octopine-positive and in Coker 201 more than 80% became embryogenic and regenerated into plants. At this writing, approximately 86 octopine-positive plantlets have been produced using different constructs. About 30% of these regenerated into normal plants. Six of the octopine-positive

plants tested showed the DNA fragments of appropriate size showing *ocs*, *NPT* II, and *bt* genes. In another experiment eight octopine-positive plants and 10 octopine-positive calli tested were positive for NPT II protein in western blot and in ELISA. All octopine-positive plantlets so far tested were kanamycin-resistant in leaf callus assay. The efficiency of this transformation-regeneration system permits the introduction of desirable genes such as insect-resistance, herbicide-resistance and virus-resistance to cotton. Cotyledon pieces (approximately 0.5 cm^2 surface area) from sterile 12 to 14 day old coker 201 seedlings were dipped in *A. tumefaciens* liquid cultures in Petri dishes and gently shaken for a few seconds to ensure contact of all cotyledon edges with the bacterial cultures. the cotyledon pieces were then blotted dry and plated on Whatman #1 filter paper on a callus initiation G_2 medium containing 0.2% "GEL-RITE" (Kelco). After three days cocultivation at low temperature (25°C) and 16 h/day photoperiod at 90 $\mu E \cdot m^{-1} \cdot s^{-1}$ light, cotyledon pieces were transferred to Petri plates without the filter paper containing the same medium supplanted with 500 mg/l carbenicillin and 25 mg/l kanamycin sulfate (both from U.S. Biochemicals).

After page 123, after Table 13, please insert the following Tables 14-19:

Table 14: Media Composition Used for Cotton Regeneration[1]

Medium	NAA (mg/l)	2iP (mg/l)	IAA (mg/l)	kinetin (mg/l)	zeatin (mg/l)	MS salts	% (w/v) glucose (g) or sucrose (s)
$1/2G_0$						1/2X	1.5g
G_0						1X	3.0g
G_1	5	1				1X	3.0g
G_2	0.1	5				1X	3.0g
G_3	5	0.1				1X	3.0g
G_4			0.1	1		1X	3.0g
G_5			2	1		1X	3.0g
EF_18	2			1		1X	3.0g
MS_{zn-g}	0.1				1	1X	1.5g
EG_1				1		1/2X	1.5s
EG_3	0.01					1/2X	0.5g

[1]All media were prepared with MS salts (Gibco, Grand Island, NY), 100 mg/l myo-inositol, 0.4 mg/l thiamine HCl, 0.2% Gel-rite (Kelco, San Diego, CA), pH 5.9. The remaining media used have already been published or are mentioned in the text. All chemicals (unless indicated) were purchased from Sigma Co. (St. Louis, MO). IAA and zeatin were filter sterilized, and the rest were autoclaved.

Table 15: Effects of different media on cotton embryogenic callus frequency of Cokers 10 weeks and GSA's 8 months after explantation. A total of 30-40 calli per cultivar were tested.

Initiation & induction media	Embryogenic calli formation medium	% Embryogenic calli[1] (% Embryogenic mass)[2]							
		Coker				GSA			
		201	208	310	315	25	71	75	78
EF18	EFs18	3(1)	0	0	2(1)	0	--	--	0
$G_2 - G_3$	½ G_0	--	--	--	--	0	3(1)	2(1)	--
$G_2 - G_3$	G_0	85(75)	49(38)	61(52)	80(68)	(4)	10(4)	12(4)	20(15)
$G_2 - G_1$	G_1	50(41)	(17)	(18)	(12)	(3)	0	--	--
$G_2 - G_3$	G_3	54(45)	(31)	(32)	(15)	(3)	(6)	(4)	--
$G_2 - G_4$	G_4	(26)	(16)	(1)	(8)	--	--	--	--
$G_2 - G_5$	G_5	(14)	--	(1)	(10)	--	--	--	--

[1]Based on numbers of calli.

[2]% Embryogenic mass was estimated as $\frac{\text{mass of embryogenic calli}}{\text{total mass of calli}}$ X 100

Table 16: Effects of different media on cotton somatic embryo[1] germination and plantlet formation. (Total number embryos are shown with percentage of embryos germinated and formed plantlets, respectively.)

Medium	Coker 201	Coker 310	Coker 315	Coker 4360	GSA78
GRM_{gn}	650 (75, 55)	30 (80, 20)	41 (46, 24)	28 (36, 25)	152 (72, 56)
EG_1	83 (30, 16)	24 (67, 17)	23 (52, 4)	26 (38, 15)	--
EG_3	175 (48, 25)	25 (68, 4)	16 (50, 6)	44 (41, 2)	--
GRM	71 (51, 30)	--	--	--	--
EG^2	43 (47, 12)	--	--	--	--

[1]Somatic embryos were selected randomly so they consisted of normal, abnormal, small and large embryos, and they were distributed randomly over different media.

[2]Embryo germination medium used by Shoemaker, R.C. *et al.*, *supra.* containing MS salts, 1 mg/l kinetin and 1.5% sucrose.

Table 17: Effects of embryo formation and embryo germination media on Coker 201 embryo germination and plantlet development.[1]

Induction medium	Embryo Formation Medium	% Normal Embryos	Embryo Germination Medium	Total Number Embryos Plated (% Germinated, % Formed Plantlets)
G_1, G_3	G_0	44	G_0	25 (24, 24)
			G_1	16 (44, 33)
			EG_3	25 (40, 32)
			GRM_{gn}	46 (63, 54)
G_1	G_1	14	G_1	23 (22, 13)
			GRM_{gn}	30 (27, 23)
G_3	G_3	32	G_0	26 (38, 19)
			G_1	16 (19, 17)
			EG_3	35 (31, 23)
			GRM_{gn}	35 (37, 37)

[1]Calli were initiated from hypocotyl sections on G_2, after three weeks transferred to G_1 or G_3, and after another three weeks were transferred to embryo formation media.

Table 18: Transformation Frequencies of Cotton Cotyledon Segments.

		Transformation Frequencies (%)			
		Growing callus in the presence of kanamycin at (μg/ml)			Octopine Positive
Construct	Cultivar	25	50	100	
pH575	Coker 201	75	78	85	33
	GSA 75	40	54	43	32
LBA4404	Coker 201	20	13	2	0
	GSA 75	14	11	0	0

Table 19.

Sample	NPTII ng/ml	protein g/ml	ngNPT/mg protein % expression
1	325.9	8926	36.6
2	209.7	11744	17.9
3	714.7	14336	49.9
4	238.2	14673	16.2
5	318.4	10470	30.4
6	349.4	9489	36.8
7	253.5	9151	27.6
8	144.1	14335	13.4
9 (neg. cntrl)	0.0	10770	0.0

After Figure 3, Please insert the attached Figure 4.

Remarks

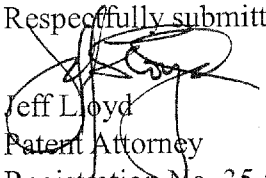
The above amendment to the Specification merely explicitly sets forth pertinent excerpts from the text of the Firoozabady application Serial No. 07/076,339 at the point it was referenced in the subject application. Tables 14-19 and Figure 4, also from Firoozabady '339, are inserted as appropriate after Tables 1-13 and Figure 3, respectively. Accordingly, no new matter is presented by this amendment. New claims 24-46 are presented and claims 20-23 are cancelled. New claim 24 is identical to what was pending at one time as claim 54 in related application serial number 08/461,240. In the '240 application, Examiner Fox indicated in his Office Action dated February 10, 2003, that claim 54 was only rejected for obviousness-type double-patenting over then-co-pending application serial nos. 08/122,322; 08/122,352; and 08/487,495; and for no other reason. Of these applications, the '352 and '495 applications have issued as U.S. Patent nos. 6,573,437 and 6,660,914 respectively. The '322 application has been abandoned. Accordingly, a terminal disclaimer over the 6,573,437 and 6,660,914 Patents is filed herewith to address that issue, and claim 54 of the co-pending '240 application has been canceled. Upon entry of this amendment claims 24-46 will be before the Examiner.

In view of the above amendments Applicants respectfully request that the suspension of prosecution of this application be lifted. In addition, Applicants are currently preparing a Petition to Correct Inventorship which has been necessitated by this amendment, and which will be filed as soon as all necessary documents have been executed.

The Commissioner is hereby authorized to charge to Deposit Account 19-0065 any fees under 37 CFR 1.16 or 1.17 as required by this paper.

Applicants invite the Examiner to call the undersigned if clarification is needed on any of this amendment, or if the Examiner believes that a telephone interview would expedite prosecution of the subject application to completion.

Respectfully submitted,



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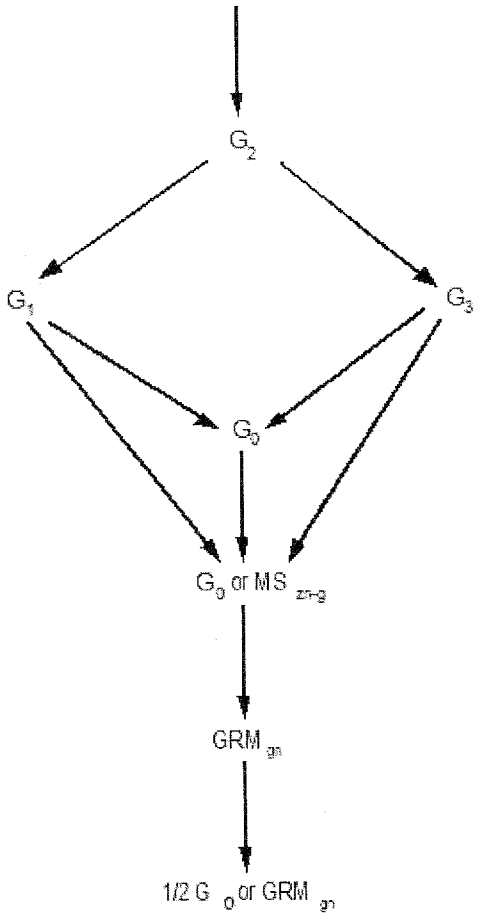
JL/ehm/amh
Attachment: Terminal Disclaimer; Figure 4

HYPOCOTYL, COTYLEDON, AND LEAF SECTIONS



TIME (DAY)		DEVELOPMENTAL STAGE
COKERS	GSAs	
		Callus Initiation
14-21	45-60	
		Embryogenic Callus Induction
28-35	140-160	
		Embryogenic Callus Formation
21-28	40-50	
		Embryo Maturation
21-28	21-28	
		Embryo Germination and Plantlet Development
7-14	7-14	
		Further Plant Development

FIG. 4

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	David T. Fox
Art Unit	:	1638
Applicants	:	Michael J. Adang, John D. Kemp
Serial No.	:	08/478,153
Conf. No.	:	6814
Filed	:	June 7, 1995
For	:	Insect Resistant Plants

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

TERMINAL DISCLAIMER

Sir:

The owner, Dow AgroSciences LLC, of 100% interest in the above-identified patent application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application, which would extend beyond the expiration date of the full statutory term defined in 35 U.S.C. 154 to 156 and 173, as shortened by any terminal disclaimer, of prior Patent Nos. 6,573,437 and 6,660,914. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term as defined in 35 U.S.C. 154 to 156 and 173 of the prior patents, as shortened by any terminal disclaimer, in the event that either of them later expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer.

☒ I am the attorney of record.

☒ I authorize the Patent Office to charge the amount of ☐ $65.00 (small entity) ☒ $130.00 (large entity) for the terminal disclaimer fee under 37 CFR 1.20(d) to Deposit Account No. 19-0065.

5 January 2006
Date

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